METHOD DEVELOPMENT AND VALIDATION OF SIMULTANEOUS ESTIMATION OF TENOFOVIR AND LAMIVUDINE I N PHARMACEUTICAL DOSAGE FORMS BY RP-HPLC

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In partial Fulfillment for the award of degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

Submitted by

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CERTIFICATE

This is to certify that the dissertation work entitled "**METHOD DEVELOPMENT AND VALIDATION OF SIMULTANEOUS ESTIMATION OF TENOFOVIR AND LAMIVUDINE IN PHARMACEUTICAL DOSAGE FORMS BY RP-HPLC**" is a bonafide work of **Mr. K.VENKATA PULLA REDDY** carried out in BIOSERVE LABORATORIES, HYDERABAD under my guidance and under the supervision of Mr. A.V. RAGHURAM Senior research Analyst and has completed to my fullest satisfaction for partial fulfillment of the award of degree of **Master of Pharmacy in Pharmaceutical Analysis**, RVS college of Pharmaceutical Sciences, Sulur, Coimbatore, which is affiliated to The Tamilnadu Dr. M.G.R Medical University, Chennai. It is to certify that the part or whole of the work has not been submitted either to this university or any other university. This work is original and confidential.

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INTERNAL EXAMINER

Place:

Date:

EXTERNAL EXAMINER

Place:

Date:

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PULLA REDDY

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1. INTRODUCTION

ANALYTICAL CHEMISTRY

Analytical chemistry[1,2] may be defined as the science and art of determining the composition of materials in terms of the elements of compound contained. By means of analytical techniques both qualitative and quantitative analysis can be done.

Analytical chemistry is basically concerned with the determination of the chemical composition of matter however, identification of substance, the elucidation of its structure and quantitative analysis of its composition are the aspects covered by modern analytical techniques.

Qualitative methods yield information about the identification of atomic or molecular species or functional groups in the sample.[3]

Quantitative analysis establishes the relative amount of one more of the species or analyte in numerical terms. Qualitative information is required before a quantitative analysis is undertaken.

Steps involved in quantitative analysis: [3,4]

- Selection of method.
- Chemical nature of sample
- Elimination of possible interferences
- Measurement of analyte
- Calculation of results
- Estimation of reliability of the results(validation)

Classification of analytical methods:

Analytical methods[5] are often classified as being either classical or Instrumental methods.

Classical methods:

The classical methods for separating and determining analyte still find use in many laboratories. For qualitative analysis the separated components were then treated with reagents that yield products that could be recognized by their colors, their boiling or melting point the solubility in a series of solvents, their odour, optical activities and their refractive indexes. For quantitative analysis the amount of analyte was determined by gravimetric or titrimetric measurements the mass of the analyte or some compounds produced from the analyte was determined. In gravimetric measurements the mass of the analyte or some compounds produced from the analyte was determined.

In titrimetric procedures the volume or mass of a standard reagent required to react completely with analyte was measured.

Instrumental methods:

In the instrumental methods[6] the measurement of physical properties of analysis such as conductivity, electrode potential, light absorption, emission. Mass to charge ratio and fluorescence began to be used for quantitative analysis of a variety of inorganic, organic & biochemical analytes.

Chromatographic and electrophoretic techniques for the separation of components complex mixtures prior to their qualitative or quantitative determination.

Analytical methods classification [3,7]

- Spectral methods
- Chromatographic methods
- Electrochemical methods
- Miscellaneous methods
- Hyphenated methods

Spectral methods:

- UV visible spectroscopy
- Atomic absorption spectroscopy
- Fluorescence and phosphorescence spectroscopy
- Infra red spectroscopy

- Nuclear magnetic resonance spectrometry
- Electron spin resonance spectroscopy
- Turbidometry
- Nephlometry
- Raman spectroscopy
- X ray diffraction etc.

Chromatographic methods

- Thin layer chromatography
- High performance liquid chromatography
- Gas chromatography
- Super critical fluid chromatography
- Paper chromatography etc...

Electrochemical methods

- Conductometry
- Potentiometry
- Coulometry
- Voltametry
- Polarography
- Amperometry
- Elecrogravimetry
- Paper electrophoresis
- Colorimetry

Miscellaneous methods

- Thermal analysis
- Mass spectrometry

Other Hyphenated methods:

- Liquid chromatography-Mass spectroscopy,[LC-MS]
- Liquid chromatography- Nuclear magnetic resonance spectroscopy,[LC-NMR]
- Gas chromatography-Mass spectroscopy[GC-MS]
- Mass spectrometry-Mass spectroscopy[MS-MS]

CHROMATOGRAPHY

Chromatography[8,9] is a technique used for the separation, purification and identification of the compounds of mixtures by their continuous distribution, between two phases. One is stationary phase and the other is mobile phase. As a general rule, highly polar materials are best separated using partition chromatography, while very nonpolar materials are separated using adsorption chromatography. Between extremes, either process might be applicable.

The Chromatographic technique was first invented by M.TSWETT in 1906.The term chromatography (Greek: - Khromatos – color and graphos –written) and its principles were first discovered by Mikhail Tswett.

On the general progress of science, chromatography may be regarded as an analytical technique employed for the purification and separation of organic and inorganic substances. It is also found useful for the fractionation of complex mixture, separation of closely related compound such as isomers and in the isolation of unstable substances.

In adsorption chromatography the adsorbent is usually kept constant, and the eluting solvent polarity is increased until elution is achieved. Some commonly used solvents in order of increasing polarity are: light petroleum solvents (hexane, heptanes, petroleum ether) < cyclohexane < toluene < dichloromethane < chloroform < ethyl ether < ethyl acetate < acetone < n-propanol < ethanol < water.

Principles of Chromatographic Separation:[10,11]

- **Adsorption chromatography:** a solid stationary phase and a liquid or gaseous mobile phase.

- **Partition chromatography:** a liquid stationary phase and a liquid or gaseous mobile phase.
- **Size exclusion chromatography**: an inert gel which acts as a molecular sieve, and liquid mobile phase.
- **Ion exchange chromatography:** a solid polymeric stationary phase containing replaceable ions.

Adsorption chromatography:

In adsorption chromatography, mixture of compounds dissolved in the mobile phase, moves through a column of stationary phase, due to the affinity of the solute towards mobile or stationary phase, Solutes get separated. Which compounds have lesser affinity elute first. In normal phase the mobile phase is non polar and the stationary phase is polar where in reverse phase the mobile phase is polar and the stationary phase is non polar.[1]

Partition chromatography:

In partition chromatography the principle of separation is readily understood by considering the partitioning behavior of substance between two immiscible liquids. Few substances, when shaken with two immiscible liquids, partition take place completely in to one or other liquid. Instead, most distribute themselves between the liquids such that the partition coefficient (the ratio of concentrations of the substance in each phase) is a constant value independent of the total amount, provided neither phase is saturated with the substance.[27]

Mode of chromatographic operations:

There are three modes of chromatographic[12,13] operation they are as follows:

- **Elution techniques**
  - Isocratic method
  - Gradient method
- Frontal techniques
- Displacement techniques

Types of chromatography techniques:

- Planar chromatography
- Column chromatography

TYPES OF LIQUID CHROMATOGRAPHY[14,15]

- Liquid – Solid chromatography
- Liquid – liquid chromatography
- Gas liquid chromatography

These three types are the basic types of chromatography and these are modified to different types of chromatography. They are as follows;

- Normal phase chromatography
- Reverse phase chromatography
- High performance liquid chromatography
- Ion exchange chromatography
- Size exclusion liquid chromatography
- Super critical fluid chromatography
- Chiral chromatography
- Affinity chromatography

Basic operations

Liquid chromatography[16,17]

1. Feed Injection:

The feed is added into the mobile phase and it is injected. The mobile phase flows through the column by the action of a pump.

2. Separation in the column:

As the sample flows through the column, its different components will adsorb to the stationary phase to varying degrees. Those with strong attraction to the support move more slowly than those with weak attraction. This is how the components are separated.

3. Elution from the column:

After the sample is flushed or displaced from the stationary phase, the different components will elute from the column at different times. The components with the least affinity from the stationary phase (the most weakly adsorbed) will elute first,

While these with the greatest affinity from the stationary phase (the most strongly adsorbed) will elute last.

4. Detection

The different components are collected as they emerge from the column. A detector analyses the emerging stream by measuring a property which is related to concentration and characteristic of chemical composition. For example the refractive index or ultra violet absorbance is measured.

High performance liquid chromatography – [HPLC]

High performance liquid chromatography (HPLC)[18] is a form of column chromatography used frequently in biochemistry and analytical chemistry. The analyte is forced through a column (stationary phase) by a liquid (mobile phase) at high pressure, and the components get separated.

Principles of HPLC:

There are two types of principles which are as follows:

- Isocratic elution
- Gradient elution

Isocratic HPLC:

In isocratic HPLC the analyte is forced through column of the stationary phase by introducing a liquid at high pressure. Use of pressure gives the components less time to diffuse within the column, leading to improved resolution in the resulting chromatogram. Solvents used include any miscible combination of water or various organic liquids (most common are methanol and acetonitrile). Water may contain buffers or salts to assist in the separation of the analyte components or compounds such as trifluroacetic acid.

Gradient HPLC:

A further refinement to HPLC is to vary the mobile phase composition during the analysis, this is known as gradient elution. A normal gradient for reverse phase chromatography might start at 5% methanol and progress linearly to 50% methanol over 25 minutes, depending on how hydrophobic the analyte is**.**

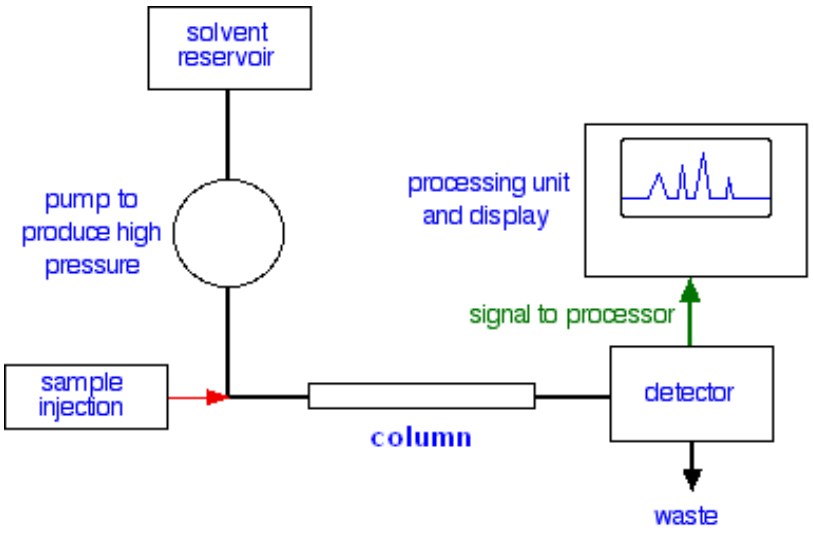
Different types of HPLC Techniques:

- Normal phase chromatography.
- Reverse phase chromatography.
- Size exclusion chromatography.
- Ion exchange chromatography.

Reverse Phase Chromatography: Reverse phase HPLC (RP-HPLC) consists of a non polar stationary phase and a polar mobile phase and was developed due to the increasing interest in large non-polar biomolecules One common stationary phase is a silica which has been treated with RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8C_{17} etc. The retention time is therefore longer for molecules which are more non-polar in nature, allowing polar molecules to elute more readily. Eluent time is increased by addition of less polar solvent.

INSTRUMENTATION OF HPLC:[6,9]

Fig No:1.1 FLOW SCHEME FOR HPLC



- Mobile phase
- Pumps
- Injector port
- Stationary phase
- Detector

MOBILE PHASE AND PUMPS:

MOBILE PHASE:

The mobile phase in RP-HPLC[16,19] however, has a great influence on the retention of the solutes and the separation of component mixture.

The primary constituents of reverse phase-mobile phase are water miscible solvents such as methanol, ethanol, acetonitrile, dioxin, and tetra hydro furan are added to adjust the polarity of the mobile phase. The water should be high quality, either distilled or demineralised. The most widely used organic modifiers are methanol, acetonitrile and tetrahydrofuran. Methanol and acetonitrile have comparable polarities but the latter is an aprotic solvent. This factor may be important if hydrogen bonding plays a significant role in the separation. When inorganic salts and ionic surfactants are used, the mobile phase should be filtered before use, since these additivies frequently contain a significant amount of water insoluble contaminants that may damage the column. Reverse phase mobile phase are generally noninflammable due to high water content. Degassing is quite important with reverse phase mobile phase.

Selection of Mobile phase:

Soluble in organic solvent:

- Non-polar compounds
  - Normal phase chromatography
  - Reverse phase chromatography
- Polar compounds
  - Non ionic

    Reverse phase chromatography

    Ion forming

    Reverse phase with pH control chromatography

Reverse phase ion paring chromatography

- Basic

  Normal phase chromatography

  Reverse phase chromatography

  Reverse phase with alkaline – eluent

Soluble in water:

* Non-ionic – Reverse phase chromatography

* Ion forming – Reverse phase with pH control chromatography

* Strong acid or bases – Reverse phase with ion pair chromatography

* Inorganic cations – Ion exchange chromatography

* Inorganic anion – Ion exchange chromatography

* Organic acids – Ion exchange chromatography

* Mono saccharides – Ion exchange chromatography

  Reverse phase chromatography

* Mono-Disaccharides– Ligand exchange chromatography

  Reverse phase chromatography

HPLC PUMPS:

Pumps system in HPLC is constructed from the materials that are inert to all mobile phase or solvent system used. The most commonly used materials are glass, stainless steel, Teflon and sapphire. The pumps are required to deliver a constant flow of mobile phase at pressure from 1 to 550 bar and 14.6 to 800 psi. Pumps capable of generating pressure up to 8000 psi provides a wide range of flow rate of mobile phase typically from 0.01 to 10ml min^{-1}.

The solvent flow from the pump should be pulse less or should be dampened in order to remove pulses since the presence of pulses in the solvent flow may cause better result with some detectors.

HPLC INJECTION PORT AND COLUMN:

INJECTION PORT FOR HPLC**:**

- Samples are injected into the HPLC via an injection port.

- The injection port of an HPLC commonly consists of an injection valve and the sample loop.
- The sample is typically dissolved in the mobile phase before injection into the sample loop.
- The sample is then drawn into a syringe and injected into the loop via the injection valve.
- A rotation of the valve rotor closes the valve and opens the loop in order to inject the sample into the stream of the mobile phase.
- Loop volumes can range between 10 µl to over 500 µl. In modern HPLC systems, the sample injection is typically automated.

STATIONARY PHASE (COLUMN):

- The stationary phase [20] in HPLC refers to the solid support contained within the column over which the mobile phase continuously flows.
- The sample solution is injected into the stationary phase through the injector port.
- As the sample solution flows with the mobile phase through the stationary phase, the components of that solution will migrate according to the non-covalent interactions of the compounds with the stationary phase.
- The chemical interactions of the stationary phase and the sample with the mobile phase, determines the degree of migration and separation of the components contained in the sample.
- Some of the more common stationary phases include: Liquid-Liquid, Liquid-Solid (Adsorption), Size Exclusion, Normal Phase, Reverse Phase, Ion Exchange, and Affinity.

- There are various columns that are secondary to the separating column or stationary phase. They are: Guard, Derivatizing, Capillary, Fast, and Preparatory Columns.

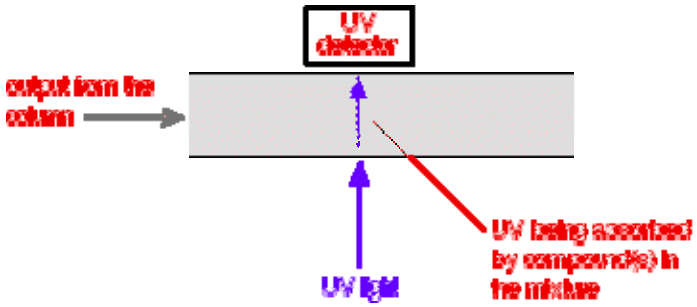
COLUMN EFFICIENCY:

- Column efficiency[21] refers to the performance of the stationary phase to accomplish particular separations.
- This entails how well the column is packed and its kinetic performance.
- The efficiency of a column can be measured by several methods which may or may not be affected by chromatographic anomalies, such as "tailing" or appearance of a "front."
- This is important because many chromatographic peaks do not appear in the preferred shape of normal Gaussian distribution.
- For this reason efficiency can be an enigmatic value since manufacturers may use different methods in determining the efficiency of their columns.

DETECTORS:

- The detector[9] for an HPLC is the component that emits a response due to the eluting sample compound and subsequently signals a peak on the chromatogram.
- It is positioned immediately posterior to the stationary phase in order to detect the compounds as they elute from the column.
- The bandwidth and height of the peaks may usually be adjusted using the coarse and fine tuning controls, and the detection and sensitivity parameters may also be controlled (in most cases).
- There are many types of detectors that can be used with HPLC. Some of the more common detectors include: Refractive Index (RI), Ultra-Violet (UV), Fluorescent, Radiochemical, Electrochemical, Near-Infra Red (Near-IR), Mass Spectroscopy (MS), Nuclear Magnetic Resonance (NMR), and Light Scattering (LS).
- Ultra-Violet (UV) detectors measure the ability of a sample to absorb light. This can be accomplished at one or several wavelengths.

- There are several ways of detecting when a substance has passed through the column. A common method which is easy to explain uses ultra-violet absorption.
- Many organic compounds absorb UV light of various wavelengths. If you have a beam of UV light shining through the stream of liquid coming out of the column, and a UV detector on the opposite side of the stream, you can get a direct reading of how much of the light is absorbed.
- The amount of light absorbed will depend on the amount of a particular compound that is passing through the beam at the time.



- One might wonder why the solvents used don't absorb UV light. They do! But different compounds absorb most strongly in different parts of the UV spectrum.
- Methanol, for example, absorbs at wavelengths below 205 nm, and water below 190 nm. If you were using a methanol-water mixture as the solvent, you would therefore have to use a wavelength greater than 205 nm to avoid false readings from the solvent.

Fixed wave length measures at one wavelength, usually 254 nm.

Variable Wavelength measures at one wavelength at a time, but can detect over a wide range of wavelengths.

Diode Array measures a spectrum of wavelengths simultaneously

UV detectors have a sensitivity to approximately 10^{-8} or 10^{-9} gm/ml.

- Calculation of column efficiency value:

All the following methods use this formula that measures N, or number of theoretical plates:

$$N = a \frac{t_r^2}{W^2}$$

a = constant dependent on height where peak width measured
t_r = retention time
W = peak width

HPLC Methods of Analysis for Drugs[14]:

Most of the drugs in multicomponent dosage forms can be analyzed by HPLC method because of the several advantages like rapidity, specificity, accuracy, precision and ease of automation in this method. HPLC method eliminates tedious extraction and isolation procedures. Some of the advantages are:

- Speed (analysis can be accomplished in 20 minutes or less),
- Greater sensitivity (various detectors can be employed),
- Improved resolution (wide variety of stationary phases),
- Reusable columns (expensive columns but can be used for many analysis),
- Ideal for the substances of low volatility,
- Easy sample recovery, handling and maintenance,
- Instrumentation tends itself to automation and quantitation (less time and less labour),
- Precise and reproducible,
- Calculations are done by integrator itself,
- Suitable for preparative liquid chromatography on a much larger scale.

QUANTITATION

Quantitation Methods in HPLC: [4, 17]

Peak height or peak area measurements only provide a response in terms of detector signal. This response must be related to the concentration or mass of the compound of interest. To accomplish this, some type of calibration must be performed.

The four primary techniques for quantitation are

1. Normalized peak area method
2. External Standard method
3. Internal Standard method
4. Method of Standard addition

1. Normalized peak area method:

The area percent of any individual peak is referred to the normalized peak area. This technique is widely used to estimate the relative amounts of small impurities or degradation compounds in a purified material and in this method; the response factor for each component is identified.

2. External Standard method:

This method includes injection of both standard and unknown and the unknown is determined graphically from a calibration plot or numerically using response factors.

A response factor (R_f) can be determined for each standard as follows.

$$R_f = \frac{\text{Standard Area (Peak height)}}{\text{Standard Concentration}}$$

The external standard approach is preferred for most samples in HPLC that do not require extensive sample preparation. For good quantitation using external standards, the chromatographic conditions must remain constant during the separation of all standards and samples. External standards are often used to ensure that the total chromatographic system is performing properly and can provide reliable results.

3. Internal Standard method:

A widely used technique of quantitation involves the addition of an internal standard to compensate for various analytical errors. In this approach, a known compound of a fixed concentration is added to the known amount of samples to give separate peaks in the chromatograms to compensate for the losses of the compounds of interest during sample pretreatment steps. Any loss of the component of interest will be accompanied by the loss of an equivalent fraction of the internal standard. The accuracy of this approach obviously dependence on the structural equivalence of the compounds of interest and the internal standard.

The requirements for an internal standard are:

a**.** It must have a completely resolved peak with no interferences,

b**.** It must elute close to the compound of interest,

c**.** It must behave equivalent to the compound of interest for analysis like pretreatments, derivative formations, etc.

d**.** It must be added at a concentration that will produce a peak area or peak height ratio of about unity with the compound,

e**.** It must not be present in the original sample,

f**.** It must be stable, unreactive with sample components, column packing and the mobile phase and

g**.** It is desirable that this compound is commercially available in high purity.

The internal standard should be added to the sample prior to sample preparation procedure and homogenized with it. To be able to recalculate the concentration of a sample component in the original sample, we have to demonstrate first the response factor. The response factor (RF) is the ratio of peak areas of sample component (A_x) and the internal standard (A_{ISTD}) obtained by injecting the same quantity. It can be calculated by using the formula,

$$Rf = \frac{Ax}{A.I.STD}$$

On the basis of the response factor and strength of the internal standard (N_{ISTD}), the amount of the analyte in the original sample can be calculated using the formula,

$$X = \frac{A_S}{R_F . A_{1STD}} x N_{1.STD}$$

The calculations described above can be used after proving the linearity of the calibration curve for the internal standard and the analytical reference standard of the compound of interest. When more than one component is to be analyzed from the sample, the response factor of each component should be determined in the calculations using similar formula.

4. Method of Standard addition:

The method of standard addition can be used to provide a calibration plot for quantitative analysis. It is most often used in trace analysis. An important aspect of the method of standard addition is that the response prior to spiking additional analytes should be high enough to provide a reasonable S/N ratio (>10), otherwise the result will have poor precision.

ANALYTICAL METHOD DEVELOPMENT[22,23]

Selecting an accurate assay procedure for each ingredient present in pharmaceutical dosage forms, either individually or complex dosage formulation containing several therapeutically and chemically compatible drugs with very similar chemical nature is a monumental undertaking.

Separation, identification and estimation of each ingredient in such complex formulation are a challenging task. The presence of excipients, additives and decomposition products further complicates the analysis. Therefore analytical development is done for new drug where no methods are available. Or, alternate method development for existing (non pharmacopoeia) products to reduce cost and time of analysis.

Method development is done for

1. New products
2. Existing products

Methods are developed for new products when no official methods are available. Alternate methods for existing (non-pharmacopoeia) products are developed to reduce the cost and time for better precision and ruggedness.

STEPS TO BE FOLLOWED IN METHOD DEVELOPMENT

Method development[22] starts with the documentation of the developed studies. All data related to these studies must be established and they must be recorded in laboratory notebook or an electronic data base.

1. Standard Analyte Characterization:-

- Collection of information about the analyte or drug should be collected starting from the structure, physical, chemical properties toxicity, purity, hygroscopic nature, solubility and stability.
- Reference standard for the sample should be obtained. In case of multiple components for the analysis to be analyzed in the sample, number of components should be noted, data is assembled and the availability of standards for each one is determined.
- Proper storage are set for the reference standards (refrigerators, dessicator, freezer).
- A suitable method for the sample is considered.

2. Method Requirements:-

The aim or requirement of the analytical method that need to be developed all considered and the analytical figures of merit are defined. The required detection limits, selectivity, linearity, range, accuracy and precision are defined.

3. Literature Search and prior Methodology:-

The literature for all types of information related to the analyte surveyed. Literature is done for synthesis, physical, chemical properties, solubility and related analytical method. Information can be obtained from official standard books such as USP/NF, AOAC standard books, periodicals, chemical manufacturers along with chemical abstract services and computer data banks.

4. Choosing of Method:-

- Adaptation is more efficient than "reinventing the wheel". If any of the reported methods from the literature are adaptable to the current laboratory setting and future needs, it is determined.
- From the various source a methodology is adopted. The methods are modified.
- If there are no reported details for the drug or the chemical then the drugs are investigated and are worked out.

5. Instrumental Setup and Initial Studies:-

- By using the data made for the analyte a suitable instrument method is selected. The instrument is set up by using standard operation procedure.
- Analyte reference standard is prepared in suitable concentration by using various combination of solvent system. It is important to start with reference standard substance rather then complex sample components.
- The analysis is made for the various conditions described in the existing literature.

6. Optimization:

During optimization one parameter is changed at a time, and set of conditions are isolated, rather than using a trial and error approach. Work has been done from an organized methodological plan, and every step is documented (in a lab note book) in case of dead ends.

7. Documentation of analytical figures:

The originally determined analytical figures of merit, Limit of quantitation [LOQ] and Limit of the detection [LOD], linearity, time per analysis cost, sample preparation etc are documented.

8. Evaluation of Method Development with actual Sample:

The sample solution should lead to unequivocal, absolute identification of the analyte peak of interest apart from all the other matrix components.

9. Determination of Percent Recovery of Actual Sample and Demonstration of Quantitative Sample Analysis:

Percent recovery for the standard analyte into a sample matrix that is shown to contain no analyte is determined. Reproducibility of recovery from sample to sample and whether the recovery has been optimized has been shown. It is not necessary to obtain 100% recovery as long as the results are reproducible and known with high degree certainty.

The validity of analytical method can be verified only by laboratory studies. Therefore documentation of the successful completion of the studies is the basic requirement for determining whether a method is suitable for its intended applications.

Validation is an approach to form a basis for written procedures for production and process control which are designed to assure that the drug products have the identity strength, quality, and purity they purport or are represented to possess.

VALIDATION OF ANALYTICAL METHOD DEVELOPMENT

Introduction:

Analytical method validation[24,25] is the process of demonstrating that analytical procedures are suitable for their intended use and provide accurate test results that evaluate a product against its defined specification and quality attributes.

It is process involving confirmation or establishing by laboratory studies the method / procedure/ system/ analyte gives accurate and reproducible result for intended application in a proven and established range. That performance characteristic of the method (accuracy, precession, sensitivity, ruggedness etc).

Definitions of validation:

World health organization (WHO): Action of providing that any procedure, process, equipment, material activity, or system actually reads to the expected results.

Food and drug administration (FDA US): Establishing documentation evidence, which provides a high degree of assurance that specific process, will consistently produce a product meeting its predetermined specification and quality attributes.

European committee(EC): action of providing in accordance with the principle of good manufacturing practice, that any procedure process equipment material, activity or system actually read to the expected result.

Types of validation:[26]

Prospective Validation: At least three successive production size (US Via) batches, all batches made tested and report approved before distribution facilities and equipment qualified.

Concurrent Validation: Generation of validation data concurrent or simultaneously with normal production schedules used in exceptional cases (low volume products); interim reports required.

Retrospective Validation: This is establishing documented evidence that the process is performed satisfactorily and consistently over time, based on review and analysis of historical data. The source of such data is production and QA/QC records. The issues to be addressed here are charged to equipment, process, specification and other relevant changes in the past.

1.9.1 VALIDATION OF ANALYTICAL PROCEDURES [19,27]

Definitions, Methodology and Acceptance Criteria:

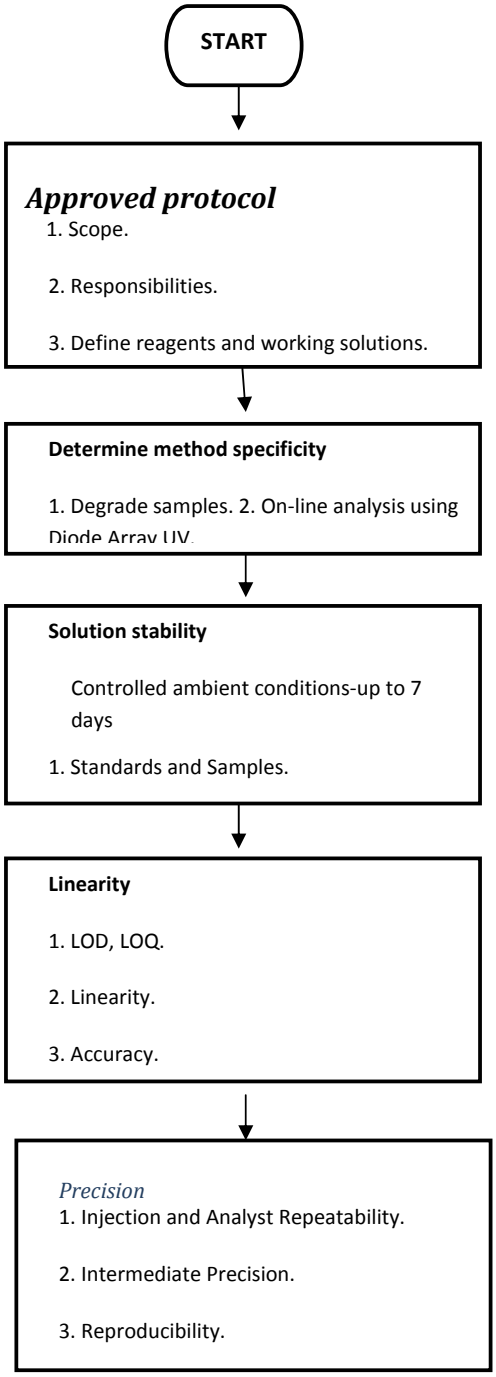
Different Types of Validation characteristics:

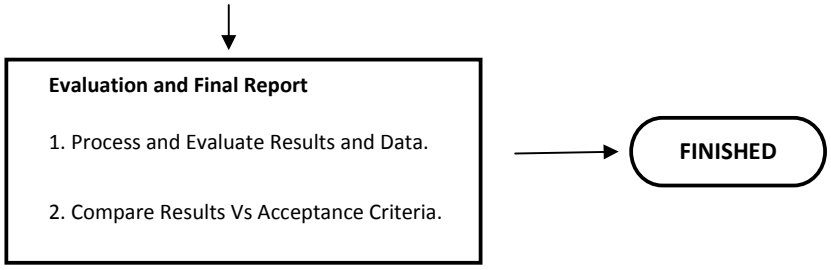
- Precision
- Accuracy
- Specificity and Selectivity
- Linearity and Range
- Solution stability
- Limit of Detection (LOD)
- Limit of Quantification (LOQ)
- Robustness
- Ruggedness.
- System Suitability

Generalized validation process for an HPLC assay method:

Validation[11] is the process of collecting documented evidence that the method performs according to its intended purpose. The validation process has been described as follows:

Table No: 1 schematic diagram of method development





1. Accuracy

Accuracy[2] is the closeness of test results obtained by that method to the true value. The accuracy of an analytical method should be established across its range. There are several methods that can be used for determining accuracy. The most common include

Analyze a sample of known concentration and compare the measurement to the true value. In this case, method accuracy is the agreement between the difference in the measured analyte concentration and the known amount of analyte added. That is the accuracy or % recovered is calculated as:

$$\frac{C_m \times 100}{C_t}$$

Where C_m is the measured concentration

C_t is the theoretical concentration.

Accuracy has also been reported as a sample is analyzed and the measured value should ideally be identical to the true value. Accuracy is represented and determined by recovery experiments. The usual range is being 10% above or [below] the expected range of claim. The % recovery was calculated using the formula,

$$\text{Percentage Recovery} = \frac{(a + b) - a}{b \times 100}$$

Where,

a – Amount of drug present in sample

b – Amount of standard added to the sample

ICH states that accuracy should be assessed using a maximum 9 determination over a minimum of 3 concentration levels covering the specified range (eg. 3 concentration /3 replicates each of the total analytical procedure).

Acceptance Criteria:

- For an assay method, mean recovery will be 100%± 2% at each concentration over the range of 80-120% of the target concentration.
- For an impurity method, mean recovery will be 0.1% absolute of the theoretical concentration or 10% relative, whichever is greater for impurities in the range of 0.1-2.5 % (V/W).

2. Precision:

Precision[25,28] of an analytical procedure expressed the closeness of agreement (degree of scatter) between a series of measurement obtained from multiple samplings of the same homogeneous sample under prescribed condition.

The precision of test method is usually expressed as the standard deviation or relative standard deviation of a series of measurements. Precision may be considered at three levels: *Repeatability*, *Intermediate Precision* and *Reproducibility*.

System precision:

A System precision was evaluated by measuring the peak response of drug for six replicate injection of the standard solution preparation as per the proposed method.

Method precision:

The method precision was determined by preparing the sample of a single batch of the drug for tablet formulation six times and analysed as per the propsed method.

Acceptance Criteria:

- Percentage Relative standard deviation (%RSD) NMT 1 % (Instrument precision)
- (%RSD) NMT -2% (Intra- assay precision)

3. Specificity: Specificity[28] is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Specificity shall be demonstrated by performing Placebo / blank interference and forced degradation studies.

a. **Blank interference:**

Blank solution is prepared and analysed as per test method.

b. **Placebo interference (In case of Drug products):**

The placebo solution equivalent to the test concentration is also prepared and analysed as per the test method.

4. **Forced Degradation studies:**

The sample is degraded forcefully under the various stress conditions like Light, heat, humidity, acid / base / water hydrolysis and oxidation to ensure the degradation ranging 1 % to 20 %.

a) *Light:* The Drug product, drug substance and placebo are exposed to UV light for about 200 watt hours / square meter and the overall illumination not less than 1.2 million Lax hours for visible light. The sample and placebo solution are prepared as per test method and analyzed.

b) *Humidity:* The Drug product, drug substance and placebo are exposed for about 80% RH at about 25°C for about one week. Prepare the sample and placebo solution as per test method and analyzed.

c) *Heat:* The Drug product, drug substance and placebo are exposed at 105°C for about 12 hours (For substance having low melting point below 10°C of its melting point). The sample and placebo solution are prepared as per test method and analyzed.

d) *Acid / Base:* The 0.1N acid or base solution of the drug refluxes the sample and placebo with 50 ml of acid / base solution for about 1 hour at 60°C. Neutralize the solution and dissolve the contents in diluents as per test method. Change the strength of acid and base or reflux time to ensure the desired degradation.

e) *Oxidation:* The sample was refluxed for 12 hour at 60°C with 1 % H_2O_2 or suitable oxidant and dissolved the contents in diluents as per test method. The reflux time was changed so as to ensure the desired degradation.

f) *Water:* The sample / placebo was refluxed with 100 ml of purified water for 12 hour at 60°C. Dissolve the contents in diluents as per test method. Change the reflux time so as to ensure the desired degradation.

Note: Based on the physic-chemical properties and literature stress conditions can be decided.

Acceptance Criteria:

- Placebo / Blank should not elute at the retention time of analyte peak and known impurity peak.
- Peak purity of analyte peak should be confirmed.
- Degradation of active analyte peak should be from 1% to 20%.[23]

5. Limit of Detection:

The limit of detection[4] is the lowest concentration of analyte in a sample that can be detected but not necessarily determined in quantitatively using a specific method under the required experimental conditions. Such a limit is expressed in terms of concentration of analyte in the sample.

Following are different approaches:

1. **Visual Evaluation Method:**

The sample solutions have to be prepared with known lowest concentrations of analyte and establish the minimum concentration at which the analyte can be reliably detected by analyzing as per test method.

a. Based on Signal to Noise Ratio Method:

The LOD can be expressed as a concentration at specified signal-to-noise (S/N) ratio obtained from samples spiked with analyte. A signal-to-noise(S/N) ratio between 3:1 and 2:1 is generally considered acceptable.

b. Based on the standard Deviation of the Response and the Slope:

- The blank solution has to be prepared as per test method and inject six times into the chromatographic system.
- Similarly the linearity solution staring from lowest possible concentration of analyte to 150 % (or as per protocol) of target concentration have to be prepared to establish the linearity curve.

The detection limit (DL) may be expressed as :

$$LOD = \frac{3.3 \times \text{Standard deviation of the response of the blank } (\sigma)}{\text{Slope}}$$

S= slope of the calibration curve of the analyte.

The slope shall be estimated from the calibration curve of the analyte.

6. Limit Of Quantitation:

The limit of quantitation[4] is the lowest concentration of the analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions quantitation limit is expressed as the concentration of analyte (eg. Percentage, parts per million) in the sample.

Following are different approaches:

a. Visual Evaluation Method:

The sample solutions with known lowest possible concentrations of analyte and establish the minimum concentration at which the analyte can be reliably quantified by analyzing as per test method.

b. Based on signal to noise ratio method :

The LOQ can be expressed as a concentration at specified signal-to-noise ratio obtained from samples spiked with analyte. A signal-to-noise ratio of 10:1 is generally considered acceptable. The ratio recognized by the ICH (1996) is a general rule. It has been stated that “the determination of LOQ is a compromise between the

concentration and the required precision and accuracy. That is, as the LOQ concentration level decreases, the precision increases.

c. Based on the standard Deviation of the Response and the Slope:

The blank solution as per test method and inject six times into the chromatographic system. Similarly the linearity solution staring from lowest possible concentration of analyte to 150% (or as per protocol) of target concentration and establish the linearity curve.

The limit of quantitation (LOQ) may be expressed as :

$$LOQ = \frac{10 \times \text{Standard deviation of the response of the blank}(\sigma)}{\text{Slope}}$$

The slope shall be estimated from the calibration curve of the analyte.

Acceptance Criteria:

- In Pharmaceutical application, the LOQ is typically set at minimum 0.05% for active pharmaceutical ingredients.
- LOQ is defined as the lowest concentration providing a RSD of 5%.
- LOQ should be at least 10% of the minimum effective concentration for clinical applications.
- Signal -to- noise ratio is 10:1.

7. Linearity and range:

Linearity: Linearity is the ability of the method to obtain test results that are directly proportional to the analyte concentration within a given range.

Range: Range of analytical procedure is the interval between the upper and lower concentration of analyte in the sample (including concentrations) for which it has been demonstrated that the analytical procedure has a sutable level of precision, accuracy, and linearity.

Measurement: A range of standards should be prepared containing at least 5 different concentration of analyte which are approximately evenly spaced and span 50-150% of the label claim.

At least 6 replicates per concentration to be studied. Plot a graph of concentration (on X-axis) Vs mean response (on Y-axis) calculates the regression equation.

Y – Intercept and correlation coefficient. Plot another graph of concentration (on X-axis) Vs response ratio (replicate response divided by concentration on Y-axis).

The range of the method is validated by verifying that the analytical method provides acceptable precision, accuracy, and linearity when applied to sample containing analyte at the extreme of the range as well as within the range.

Acceptance criteria:

Coefficient of correlation should be NLT 0.99.

8. Ruggedness:

Degree of reproducibility of test results obtained by the analysis of the same sample under a verity of conditions, such as different laboratories, different analysts, different instruments etc.

Normally expressed as the lack of influence on test results of operational and environmental variable of the analytical method.

Ruggedness is a measure of reproducibility of test results under the variation in condition normally expected from laboratory to laboratory and from analyst to analyst

The following are the typical method parameters need to tested during method validation:

- Analyst-to-Analyst variability.
- Column-to-Column variability.
- System-to-System variability.
- Different days.
- Different Laboratories.
- Stability of Solutions and mobile phase. (At least for 48 hours)

9. Robustness:

Robustness of an analytical method is a measure of its capacity ti remain un affectedly small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

For example a chromatographic method, the typical method parameters need to change deliberately and verify during method validation:

Flow rate	:	(+/- 0.2ml/minutes).
Mobile phase composition	:	(+/- 10% of organic phase).
Column oven temperature	:	(+/- 5°C).
P_H of buffer in mobile phase	:	(+/- 0.2 units).
Filter suitability	:	(At least two filters).

For Variations:

1. System suitability should meet the acceptance criteria as per test method.
2. If system suitability doesn't meet, the variation range is narrowed and carried out the experiment again to meet system suitability.

10. SYSTEM SUITABILITY TESTING

System suitability testing[14] is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics analytical operation and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated.

System Suitability Parameters

- Tailing factor
- Theoretical plate number
- Theoretical plate per meter

- Resolution factor
- Capacity factor
- Precision / Injection repeatability
- Relative retention

a. Tailing factor:

Asymmetry factor[24] of a peak was calculated from the following expression:

$$\text{Peak tailing factor} = \frac{A + B}{2A}$$

A – Left half of the peak at 5% peak height when the peak is bisected with a perpendicular line dropped from the maximum of peak interest.

B – Right half of the peak at 5% peak height when the peak is bisected with a perpendicular line dropped from the maximum of peak interest.

b. Theoretical plate number (N):[24]

The assessment of performance of column efficiency of a column is in terms of number of theoretical plate

An equation shown below:

$$\mathbf{N = 5.54[t/wh/2]}$$

Where,

t = Retention time

Wh/2 = width of peak at half weight

Theoretical plate number is a measure of column efficiency. i.e. how many peaks can be located per unit run time of the chromatogram ,'H' or 'HETP', the height equivalent to the theoretical plate, measures the column efficiency per unit length of the column. Parameters which can affect 'N' or 'H' include peak position particle size in column, flow rate of mobile phase and molecular weight of the analyte.

c. Capacity factor:[22]

It reflects the location of peak of interest with respect to the void volume i.e. elution time of the unretained compound.

$$K_1 = (t_r - t_o) - t_o$$

[t_r – retention of analyte, t_o – retention of void or unretained component.]

Methods used for the examination of pharmaceutical material may be broadly classified as;

d. Resolution: [27]

$$R = 2\,[(t_2 - t_1)/\, w_2 + w_1]$$

t_2, t_1 are the retention time

w_2, w1 are the peak width at base line respectively.

Resolution is to measure how well the resulting two peaks are separated. For reliable quantitation well separated peaks are essential for quantitation. This is a very useful parameter if potential interference peak may be of concern. The closest eluting peak to the analyte should be selected.

System Suitability Parameters and Recommendations (ICH Guidelines)

Parameter	**Recommendation**
Capacity Factor (k')	the peak should be well-resolved from other peaks and the void volume, generally k'>2.0
Repeatability	RSD </= 1% for N >/= 5 is desirable.
Relative retention	not essential as long as the resolution is stated.
Resolution (R_s)	R_s of > 2 between the peak of interest and the closest eluting potential interferent (impurity, excipients, degradation product, internal standard, etc).
Tailing Factor (T)	T of </= 2
Theoretical Plates (N)	In general should be > 2000

Class A: Test designed to establish identity, whether of bulk drug substances or particular ingredient in a finished dosage form.

Class B: Method designed to detect and quantitative impurities in a bulk drug substance or finished dosage form.

Class C: Methods used to determine quantitatively the concentration of a bulk drug substance or of a major ingredient in a finished dosage form.

Class D: Methods used to assess the characteristics of finished dosage forms such as dissolution profile and content uniformity.

Table No: 2 Characteristics that should be considered for different Type of analytical procedure:-

S.No	Parameters	Class A	Class B		Class C	Class D
			Quantitative test	Limit test		
1.	Accuracy	-	Yes	-	Yes	Yes
2.	Precision	-	Yes	-	Yes	Yes
3.	Robustness	Yes	Yes	Yes	Yes	Yes
4.	Linearity and Range	-	Yes	-	Yes	Yes
5.	Selectivity	Yes	Yes	Yes	Yes	Yes
6.	LOD	Yes	Yes	Yes	-	-
7.	LOQ	-	Yes	-	-	-

2. LITERATURE REVIEW

1. ***Anindita Behera et al*** [29]**.,** (2011) have down Development and validation of Spectrophotometric method for determination of Emtricitabine and Tenofovir Disoproxil Fumarate in Bulk and Tablet dosage form. Three simple Spectrophotometric methods have been developed for simultaneous estimation of Emtricitabine and Tenofovir Disoproxil Fumarate from tablet dosage form. Method A is Least Square method, involves the measurement of Emtricitabine and Tenofovir Disoproxil Fumarate at their λmax at 281.0 nm and 260.5nm respectively. Method B is First order derivative spectroscopy, wavelength selected for quantitation were 234.5nm for Emtricitabine (zero cross for Tenofovir Disoproxil Fumarate) and 281.0nm for Tenofovir Disoproxil Fumarate (zero cross for Emtricitabine). Method C is Area under Curve method, AUC in the range of 278.0- 283.0nm (for Emtricitabine) and 258.0- 262.0nm (for Tenofovir Disoproxil Fumarate) were selected for the analysis. The linearity lies between 5-25µg/ml and 10–50µg/ml for Emtricitabine and Tenofovir Disoproxil Fumarate respectively
   for method A, B and C.

2. ***Rajesh Sharma[1] et al*** [30]**.,** (2009) have developed RP- HPLC Method for Simultaneous Estimation of Emtricitabine and Tenofovir Disoproxil Fumerate in a Tablet Dosage Form. The estimation was carried out on Luna C18 (25cm x 4.60 mm, particle size 5µm) column with a mixture of acetonitrile: potassium dihydrogen phosphate buffer (pH 3.0 ± 0.05 adjusted with orthophosphoric acid): triethylamine in the ratio of 70:30:0.5(v/v) as mobile phase. UV detection was performed at 260 nm. The method was validated for linearity, accuracy, precision, specificity and sensitivity as per ICH norms. The developed and validated method was successfully used for the quantitative analysis of commercially available dosage form. The retention time was 1.78 and 2.27 min. for emtricitabine and tenofovir disoproxil fumarate respectively and total run time was 4 min. at a flow rate of 1.5 mL min-1. The calibration curve was linear over the concentration range of 5-50 µg mL-1 for emtricitabine and 5-50 µg mL-1 for tenofovir disoproxil fumarate. The LOD and LOQ values were found to be 0.015 and 0.045 µg mL-1 for emtricitabine and 0.039 and 0.117 µg mL for tenofovir disoproxil fumarate respectively. The high percentage of recovery and low percentage

coefficient of variance confirm the suitability of the method for the simultaneous estimation of emtricitabine and tenofovir disoproxil fumarate in tablet dosage form

3. ***Shirkhedkar Atul A* et al**[31]., (2009) have work down application of uv-spectrophotometric methods for estimation of Tenofovir disoproxil fumarate in tablets. Tenofovir disoproxil fumarate was estimated at 260 nm in 0.1N HCl. It showed amplitude at 273 nm, In both the methods linearity was found to be in the range of 5 - 40 µg/ml. UV- Spectrophotometric method (Y=0.02586 X+0.0083; r2=0.9999) and for first order derivative spectrophotometric method (Y=0.00132 X+0.00035; r2=0.9995), respectively. These methods were tested and validated for various parameters according to USP guidelines. The quantitation limits were found to be 1.546 and 1.986µg/ml, for both the methods.

4. ***Manish Yadav* et al** [32]**.,** (2010) have done Selective Determination of Antiretroviral Agents Tenofovir, Emtricitabine, and Lamivudine in Human Plasma by a LC-MS-MS Method for a Bioequivalence Study in Healthy Indian Subjects. The chromatographic separation is achieved in a run-time of 3.0 min on an ACE 5 CN column under isocratic conditions. The mobile phase consisted of 0.5% formic acid in water and acetonitrile (55:45, v/v). The protonated precursor product ion transitions for TFV, FTC, 3TC, and internal standard were monitored on a triple quadrupole mass spectrometer operating in the multiple reaction monitoring (MRM) and positive ion mode. A linear dynamic range of 4.0-802 ng/mL, 15.0-3006 ng/mL, and 20.1-4023 ng/mL is established for TFV, FTC, and 3TC, respectively, using 0.2 mL plasma sample. The method is fully validated for its sensitivity, selectivity, accuracy and precision, ion suppression, matrix effect, recovery, stability, and dilution integrity. It is successfully applied to a bioequivalence study of [300(TFV) + 200(FTC) + 300(3TC)] mg tablet formulation in 43 healthy human subjects under fasting conditions.

5. ***Narendra Devanaboyina* et al.,**[33] work down in hplc method development and validation for simultaneous estimation of tenofovir and emtricitabine in combined

pharmacetuical dosge form. Chromatography was carried out by using Chromosil C-18 column internal diameter with a mixture of methanol, acetonitrile and TEA in the ratio of 46:50:04 (v/v/v) as mobile phase. Determination of the different analytical parameters such as linearity, precision, accuracy, and specificity, limit of detection (LOD) and limit of quantification (LOQ) was done. The calibration curve was found to be linear for each analyte in the desired concentration range. The % recovery was found to be 99.59 and 99.61 for Tenofovir and Emtricitabine respectively. The proposed method is highly sensitive, precise and accurate, which was evident from the LOD value of 0.05 and 0.02 ppm for Tenofovir and Emtricitabine respectively and hence the present method can be applied successfully for the quantification of active pharmaceutical ingredient (API) content in the combined formulations of Tenofovir and Emtricitabine.

6. ***P.Chandra*** **et al.,**[34] have done application of high-performance thin-layer chromatographic method for the simultaneous determination of lamivudine and tenofovir disoproxil fumarate in pharmaceutical dosage form The separation was carried out on Merck HPTLC aluminum plates of silica gel 60 $F_{254,}$ with 250 µm thickness using chloroform: methanol: toluene (8: 2: 2, v/v/v) as mobile phase. HPTLC separation of the two drugs followed by densitometric measurement was carried out in the absorbance mode at 265 nm. The drugs were satisfactorily resolved with R_f values of 0.27± 0.01 and 0.51± 0.01 for LAM and TDF, respectively. The linear regression analysis data for the calibration plots showed good linear relationship with r^2=0.9999 and 0.9996 for Lamivudine and Tenofovir disoproxil fumerate respectively in the concentration range of 60-210 ng $spot^1$ for each drug.

7. ***R.Sharma*** **etal**[35]**.,**(2010) developed a Simultaneous spectrophotometric estimation of tenofovir disoproxil fumarate and lamivudine in three component tablet formulation containing efavirenz. The absorption maxima of the drugs were found to be 247, 259 and 272 nm, respectively for efavirenz, tenofovir disoproxil fumarate and lamivudine in methanol : water (50:50) solvent system. Efavirenz, tenofovir disoproxil fumarate and lamivudine obeyed Beer's law in the concentration range of 10-60, 5-30, and µg/ml,

respectively. Results of analysis for all the three methods were analysed and validated for various parameters according to ICH guidelines.

8. ***Ramesh.J et al*** [36] **.,** have done Simultaneous Estimation of Lamivudine and Tenofovir Disoproxil Fumerate in Pure and in Tablet Formulation by First Derivative Spectrophotometric Method. The solutions of standard and sample were prepared in methanol. Quantitative determination of the drugs was performed at 261 nm and at 249 nm for Lamivudine and Tenofovir disoproxil fumerate, respectively. Proposed method was evaluated for the different validation parameters. The specificity test showed that there was no interference from excipients commonly found in the commercial pharmaceutical formulations at the analytical wavelengths of Lamivudine and Tenofovir disoproxil fumerate. Quantification was achieved over the concentration range of 5 – 30 µg/ ml for Lamivudine and for Tenofovir disoproxil fumerate respectively. The correlation coefficient of Lamivudine and Tenofovir disoproxil fumerate was found to be 0.9991 and 0.9992 respectively.The mean recovery was 100.21and 98.96 % for Lamivudine and Tenofovir disoproxil fumerate, respectively. This method is simple, precise, and sensitive and applicable for the simultaneous determination of Lamivudine and Tenofovir disoproxil fumerate in pure powder and formulation.

9 ***Malipatil S M et al*** [37]**., (2009)** have reported the determination of Tenofovir Disoproxil Fumarate by a sensitive simple isocratic RP-HPLC method using Luna C18 column, mobile phase is 0.1%formic acid: acetonitrile (50:50), flow rate is 0.8ml/min with UV detection is 305nm.

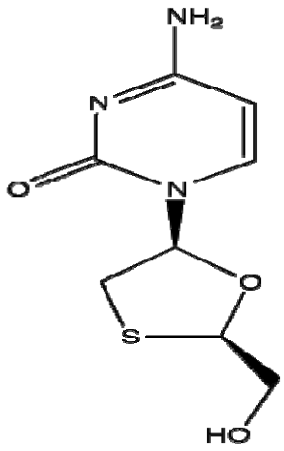
10. ***S. Sentenac, et al*** [38]**.,** *(*2003) have done the Sensitive determination of tenofovir in human plasma samples using reversed-phase liquid chromatography. A solid–liquid extraction procedure was coupled with a reversed-phase HPLC system. The system requires a mobile phase containing Na_2HPO_4 buffer, tetrabutylammonium hydrogen sulfate and acetonitrile for different elution through a C_{18} column with UV detection. The method proved to be accurate, precise and linear between 10 and 4000 ng/ml.

11. ***Alain Pruvost* et al[39].,** have done Pilot Pharmacokinetic Study of Human Immunodeficiency Virus-Infected Patients Receiving Tenofovir Disoproxil Fumarate (TDF) Investigation of Systemic and Intracellular Interactions between TDF and Abacavir, Lamivudine, or Lopinavir-Ritonavir— Liquid chromatography-tandem mass spectrometry was used to measure NRTIs and NRTI-TPs. Statistical analyses were performed on pharmacokinetic parameters: the area under the concentration-time curve from 0 to 4 h , the maximum concentration of the drug (C_{max}), and the residual concentration of the drug at the end of the dosing interval (C_{trough}) for plasma and the AUC_{0-4} and C_{trough} for intracellular data.

3. DRUG PROFILE

3.1 LAMIVUDINE[32]

Molecular Structure :



Lamivudine

Molecular Formula : $C_8H_{11}N_3O_3S$

Chemical Name : 4-amino-1-[(2*R*,5*S*)-2-(hydroxyl methyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one

Molecular Weight : 229.26 g/mol

Category : Anti-HIV Agents

Nucleoside and Nucleotide

Reverse Transcriptase Inhibitors

Physical Properties of Lamivudine

Color : White crystalline solid

Melting Point : 160-162^{o}C

State : Tablet

Solubility : Aqueous solubility - 70mg/ml at 20^{0} c

Highly Soluble in 0.01 N Hcl

Pharmacological Properties

Dosage Form : Tablet

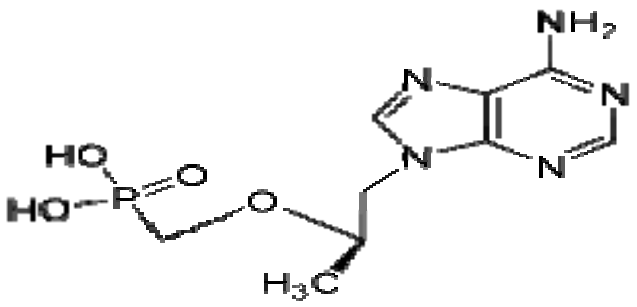
Route of administration : Oral

Pharmacology : Lamivudine was rapidly absorbed after oral administration in HIV-infected patients. Absolute bioavailability in adults is 86% ± 16% for the tablet and 87% ± 13% for the oral solution

Mechanism of Action : Lamivudine is an analogue of cytidine. It can inhibit both types (1 and 2) of HIV reverse transcriptase and also the reverse transcriptase of hepatitis B. It is phosphorylated to active metabolites that compete for incorporation into viral DNA. They inhibit the HIV reverse transcriptase enzyme competitively and act as a chain terminator of DNA synthesis. The lack of a 3'-OH group in the incorporated nucleoside analogue prevents the formation of the 5' to 3'phospho di ester linkage essential for DNA chain elongation, and therefore, the viral DNA growth is terminated.

3.2 TENOFOVIR [29]

Molecular Structure:



Tenofovir disoproxil fumarate

Molecular Formula : $C_9H_{14}N_5O_4P$

Chemical Name : ({[(2*R*)-1-(6-amino-9*H*-purin-9-yl)propan-2-yl]oxy} methyl) phosphoric acid

Molecular Weight : 287.213 g/mol

Category : Anti-HIV Agents

Nucleoside and Nucleotide

Reverse Transcriptase Inhibitors

Physical Properties of Tenofovir

Color : White crystalline

Melting Point : 276-280^oC

State : Tablet

Solubility **:** Slightly soluble in water, soluble in methanol, sparingly soluble in acetone, very slightly soluble in methylene chloride.

Pharmacological Properties

Dosage Form **:** Tablet

Route of administration **:** Oral

Pharmacology : Tenofovir exhibits longer serum (7 hours) and intracellular (>60 hours) half – lives than those of nucleoside analogues, which supports a flexible once – daily administration schedule. The pharmacokinetics of Tenofovir are dose – proportional and similar in healthy volunteers and HIV – infected individuals. The oral bioavailability of Tenofovir is enhanced by administration with a high – fat meal, but is similar at steady state when administered with or without a typical meal**.**

Mechanism of Action : Before phosphorylation, tenofovir disoproxil fumarate is converted to tenofovir in the intestinal lumen and plasma by di ester hydrolysis. Tenofovir is then internalized into cells, possibly by endocytosis, and subsequently phosphorylated in sequential steps to tenofovir monophosphate and to the active metabolite, tenofovir di phosphate. In a mechanism similar to that of NRTIs, tenofovir di phosphate competes with its natural nucleotide counterpart, deoxy adenosine 5´-triphosphate, for incorporation into newly forming HIV DNA. Once successfully incorporated, termination of the elongating DNA chain ensues, and DNA synthesis is interrupted. Although the end result of tenofovir activity is similar to that of the NRTIs, subtle differences exist between this class of drugs and tenofovir. Like tenofovir, NRTIs also must be phosphorylated to active metabolites to inhibit reverse transcription of RNA into DNA. However, since tenofovir already contains a phosphate group attached to the adenine base, the initial addition of a phosphate group is circumvented; thus, only two steps are required in the phosphorylation of tenofovir, as opposed to three with the NRTI class.

4. AIM AND OBJECTIVE

- Lamivudine is a nucleoside reverse transcriptase inhibitor. It can inhibit both types of HIV reverse transcriptase and also the reverse transcriptase of Hepatitis B. Tenofovir Disoproxil Fumarate is fumaric acid salt of the bis isopropoxy carbonyl oxy methyl ester derivative of tenofovir. Nucleotide reverse transcriptase inhibitors used in combination for the treatment of HIV infection.
- Literature survey reveals that Tenofovir Disoproxil fumerate and Lamivudine is estimated individually by UV, derivative – HPLC, Plasma RP-HPLC and Plasma LC/MS/MS methods. Few RP-HPLC methods were reported for estimation of Emtricitabine, Tenofovir and efavirenz in pharmaceutical formulation.
- RP-HPLC, LC-MS/MS and HPTLC methods were reported for the simultaneous estimation of Emtricitabine and Tenofovir disoproxil fumerate in human plasma and in formulations. Also UV, HPLC, LC–MS, HPTLC and enzymatic assay methods were reported for the simultaneous estimation of Lamivudine with other antiretero viral drugs.
- To the best of our knowledge, there is no reported RP-HPLC method for Simultaneous Estimation of Lamivudine and Tenofovir disoproxil fumerate in pharmaceutical formulations, previous to our work.
- Thus, efforts were made to develop fast, selective and sensitive analytical method for Simultaneous estimation of Lamivudine and Tenofovir disoproxil fumerate in their combined dosage form.

5. PLAN OF WORK

Aimed to develop Simultaneous estimation method and validation for Lamivudine and Tenofovir in Pharmaceutical dosage form.

The plan of the proposed work includes the following steps:

- The extensive survey of literature for Lamivudine and Tenofovir regarding their physico-chemical properties and analytical methods. This forms the basis for the development of methods..
- Selection of suitable solvent for quantitative extraction of analyte present in the formulations. The solvent should be readily available, economical and of analytical grade.
- To develop initial chromatographic conditions by selection of suitable column and appropriate wavelength in UV for detection and optimization of the method.
- To validate analytical method developed as per the ICH Q2B guidelines.

6. MATERIALS AND INSTRUMENTS

6.1 Instruments:

Table No 3: Table showing list of the Instruments used

S. No	Name of the Instrument	Make	Model
1.	HPLC	Water	Alliance-2695 PDA Waters-2996
2.	Electronic balance	Shimadzu	AY 220
3.	Digital pH meter	Digisun Electronics	7007
4.	Centrifuge	Thermolab	R_8C
5.	PhotoStability Chamber	Thermolab	943/03/0607

6.2 Reagents and chemicals:

Table No 4: Table showing list of the chemicals used

S. No	Name	Grade	Manufacturer/ Supplier
1.	Lamivudine & Tenofovir working standard	-	
2.	Acetonitrile	HPLC	Merck
3.	Potassium di hydrogen ortho phosphate	HPLC	Merck
4.	Methanol	HPLC	Merck
5.	Water	-	-
6.	Milli Q Water	HPLC	-

7. EXPERIMENTAL PART

7.1 METHOD DEVELOPMENT AND OPTIMIZATION OF LAMIVUDINE AND TENOFOVIR

1. SELECTION OF DETECTOR WAVE LENGTH:

The wave length selection is made at 260 nm since all the two compounds maximum absorbance in UV spectrum as reported in the literature is in 260 nm.

2. OPTIMIZED CHROMATOGRAPHIC CONDITIONS:

a. Selection of mode of separation:

As the drug was polar in nature, RP-HPLC method was preferred.

Procedure:

Preparation of TEA buffer:

Dissolve 1ml of TEA (tri methyl amine) into a 250ml beaker with HPLC water. Adjusted the pH to 3.0 with ortho phosphoric acid.

Preparation of mobile phase:

Mix a mixture of above buffer 400mL (40%) and 600 mL of Methanol HPLC (60%) and degas in ultrasonic water bath for 5 minutes. Filter through 0.45 µ filter under vacuum filtration.

Preparation of Standard Solution:

Accurately 10 mg of Lamivudine & Tenofovir were weighed and transferred into 10ml volumetric flask, about 7ml of diluent was added and sonicated for 5 minutes to dissolve it. The volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter (Stock solution).

From this 0.75ml of solution was pipette out and transferred into 10ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Inject 20 µl of the standard solution into the chromatographic system and measure the area for the Lamivudine & Tenofovir peaks and calculate the %Assay by using the assay formula.

TRIAL&ERROR METHODS:

TRIAL -1:

CHROMATOGRAPHIC CONDITIONS:

Column : Symmetry C8 (4.6 x 150mm, 5 µm, Make: ACE)

Detector : 260nm

Flow rate : 0.6ml/min

Injection volume : 20µl

Run time : 10min

Mobile Phase : Buffer (pH: 5.0): Acetonitrile: Methanol (30: 40: 30)

Results of Trial-1: Peak shape was not good, Due to asymmetry in peaks and longer RT's another trial is made with change in mobile phase-buffer pH.

TRIAL- 2:

CHROMATOGRAPHIC CONDITIONS:

Column : Symmetry C8 (4.6 x 150mm, 5 µm, Make: ACE)

Detector : 260nm

Flow rate : 0.7ml/min

Injection volume : 20µl

Run time : 10min

Mobile phase : Buffer (pH-.4): methanol (50:50)

Results of Trial-2: Peak shape was not good, Due to tailing in peaks and asymmetry, so reduce RT's another trial is made with change in mobile phase-buffer pH and flow rate.

Trail-3:

CHROMATOGRAPHIC CONDITIONS:

Column : Symmetry C8 (4.6 x 150mm, 5 µm, Make: ACE)

Detector : 260nm

Flow rate : 0.8ml/min

Injection volume : 20µl

Run time : 10min

Column temperature : 30°c

Mobile phase : Buffer (pH-.3): methanol (60:40)

Results of Trial-3(Final Optimized Method): Peak shape was good but second compound have more tailing factor so reduce RT's another trail is made. The peaks are sharply resolved with less tailing and hence the trial-3 method is optimized for analysis.

Final method:

Column : Symmetry C8 (4.6 x 150mm, 5 µm, Make: ACE)

Detector : 260nm

Flow rate : 0.8ml/min

Injection volume : 20µl

Run time : 10min

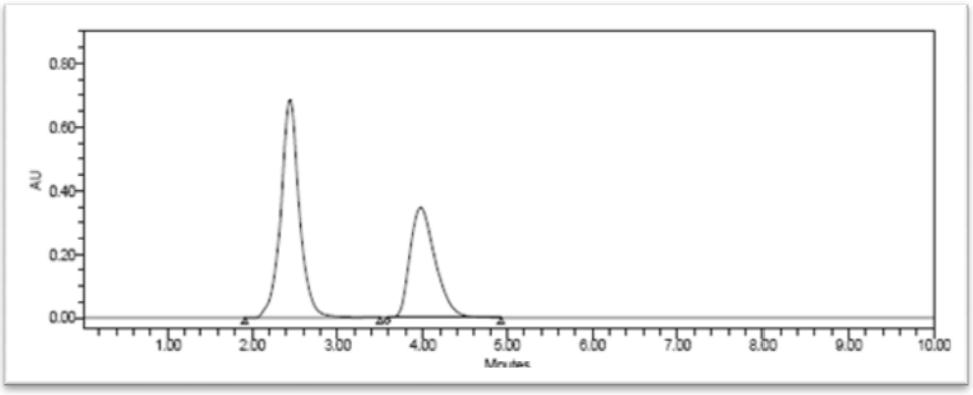
Column temperature : 30°c

Mobile phase : Buffer (pH-.3): methanol (40:60)

Results of Trial-4(Final Optimized Method): RT's were observed at 2.448 (Lamivudine), and 3.993 (Tenofovir). The peaks are sharply resolved with less tailing and hence the trial-4 method is optimized for analysis.

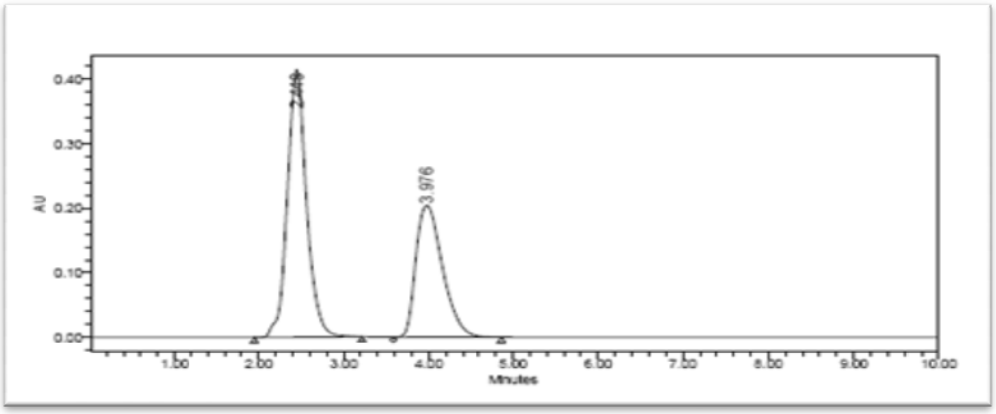
Trail-1

Fig No: 2.1



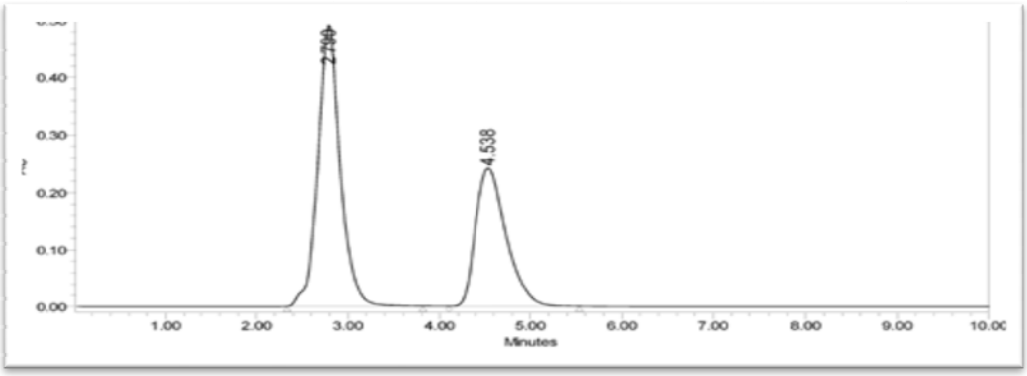
Trail – 2

Fig No: 2.2



Trail -3

Fig No: 2.3



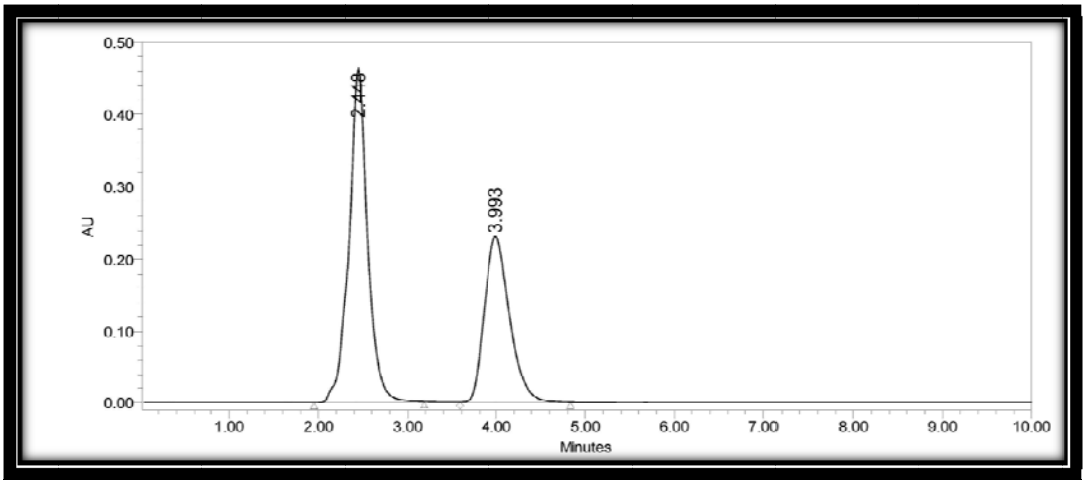
7.2 ANALYTICAL METHOD-OPTIMISATION

Aim: The present study is to develop a new reverse phase liquid chromatographic method for simultaneous determination of Lamivudine & Tenofovir in tablet dosage form.

Table No : 5 Table showing Optimized Chromatographic Parameters

OPTIMIZED CHROMATOGRAPHIC CONDITIONS	
Mode of separation	isocratic elution
Mobile phase	Solvent-A: Phosphate buffer pH-3 Solvent-B: methanol
Column	Symmetry C8 (4.6 x 150mm, 5 µm, Make: ACE)
Flow rate	0.8 mL/ min
Detection Wavelength	260 nm
Injection volume	20 µl
Column oven temperature	Ambient(30°C)
Run time	10 min

Fig No: 2.4 Chromatogram showing peaks of standard solution of Lamivudine and Tenofovir



	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Lamivudine	2.45	6739592	463588	2736.06	1.0
2	Tenofovir	3.99	4637107	231647	2853.19	1.3

7.3 QUANTITATIVE DETERMINATION OF THE DRUG BY USING THE DEVELOPED METHOD

Sample : Lamivudine & Tenofovir

Label claim : 300mg

Standard Solution Lamivudine & Tenofovir:

Accurately 10 mg of Lamivudine & Tenofovir were weighed and transferred into 10ml volumetric flask, about 7ml of diluent was added and sonicated for 5 minutes to dissolve it. The volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter (Stock solution).

From this 0.75ml of solution was pipette out and transferred into 10ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Inject 20 µl of the standard solution into the chromatographic system and measure the area for the Lamivudine & Tenofovir peaks and calculate the %Assay by using the assay formula.

Sample Solution Lamivudine & Tenofovir:

20 Lamivudine & Tenofovir tablets were weighed and the average weight was calculated. Accurately the sample equivalent to10 mg of Lamivudine & Tenofovir was weighed & transferred into 10ml volumetric flask about 7ml of diluent was added and sonicated for 5 minutes to dissolve it content. The volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter (stock solution).

0.75ml of stock solution was pipetted out and transferred into 10ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Assay formula:

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \text{average weight of tablet}$$

Where,

AT = Peak Area of sample solution.

AS = Peak Area of standard solution.

WS = Weight of working standard taken in mg

WT = Weight of sample taken in mg

DS = Dilution of Standard solution

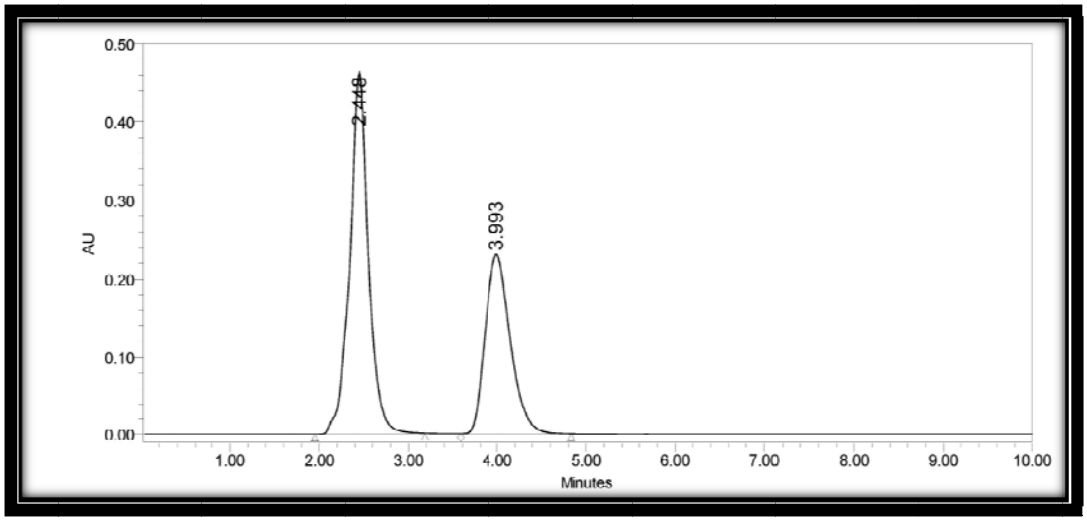
DT = Dilution of sample solution

Acceptance criteria: The limit of assay is in between the 98% - 102%

The chromatograms are as shown in Fig No: 2.5 & 2.6 and assay results are tabulated and are as shown in Table No: 6

Chromatogram showing peaks of standard solution of Lamivudine & Tenofovir.

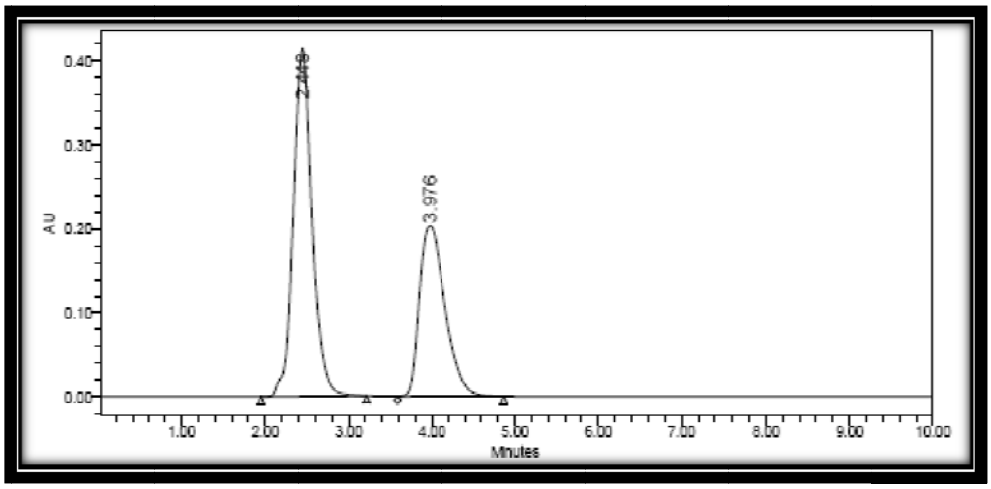
Fig No 2.5



	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Lamivudine	2.45	6739592	463588	2736.06	1.0
2	Tenofovir	3.99	4637107	231647	2853.19	1.3

Chromatogram showing peaks of test solution of Lamivudine & Tenofovir.

Fig No : 2.6



	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Lamivudine	2.448	6709473	454296	2796.42	1.1
2	Tenofovir	3.976	4598839	230126	2895.13	1.4

Table showing Assay Results of Lamivudine & Tenofovir:

Table No: 6

S. No	Compound name	Assay value
1.	Lamivudine	99.56%
2.	Tenofovir	99.1%

8. RESULTS AND DISCUSSION

8.1 VALIDATION

Definition: Validation is a process of establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce meeting, its predetermined specifications and quality attributes.

The objective of the analytical procedure should be clearly understood since this will govern the validation characteristics which need to be evaluated. Typical validation characteristics which should be considered are listed below.

- Accuracy
- Precision
- Specificity
- Linearity & Range
- Robustness
- Ruggedness
- System suitability testing

After method development, the validation of the current method has been performed in accordance with USP requirements for assay determination (Category-I: analytical methods for quantitation of active ingredients in finished pharmaceutical products) which include accuracy, precision, selectivity, linearity and range, robustness and ruggedness.

8.2 SYSTEM SUITABILITY TESTING

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated and the parameters like tailing factor, retention time, theoretical plates per unit, resolution factor are determined and the results are tabulated and are as shown in Table No: 7

Method

Preparation of Standard solution:

Standard Solution Lamivudine & Tenofovir:

Accurately 10 mg of Lamivudine & Tenofovir were weighed and transferred into 10ml volumetric flask, about 7ml of diluent was added and sonicated for 5 minutes to dissolve it. The volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter (Stock solution).

From this 0.75ml of solution was pipetted out and transferred into 10ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Inject 20 µl of the standard solution into the chromatographic system and measure the area for the Lamivudine & Tenofovir peaks and calculate the %Assay by using the assay formula.

Acceptance criteria:

a. The column efficiency is not less than 2000 theoretical plates.
b. The tailing factor for the analyte peak is not more then 2.0.
c. The relative standard deviation for the replicate injections more than 2.0%.

Chromatograms showing system suitability testing of Standard Solutions of Lamivudine & Tenofovir.

Fig No : 3.1

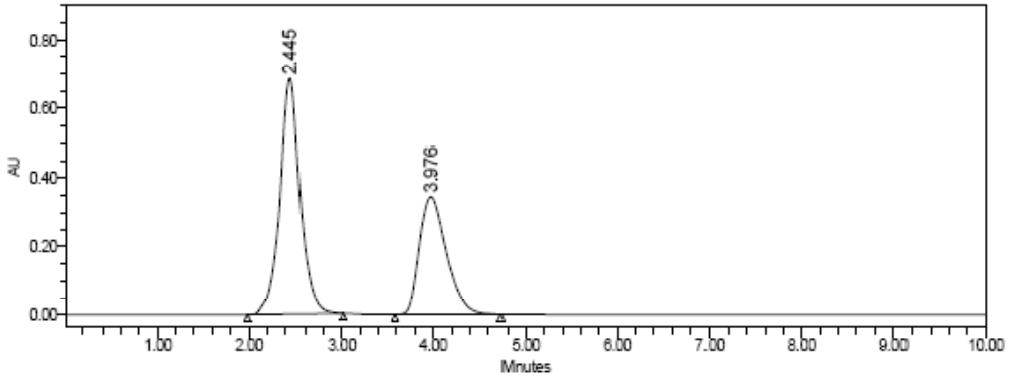


Fig No : 3.2

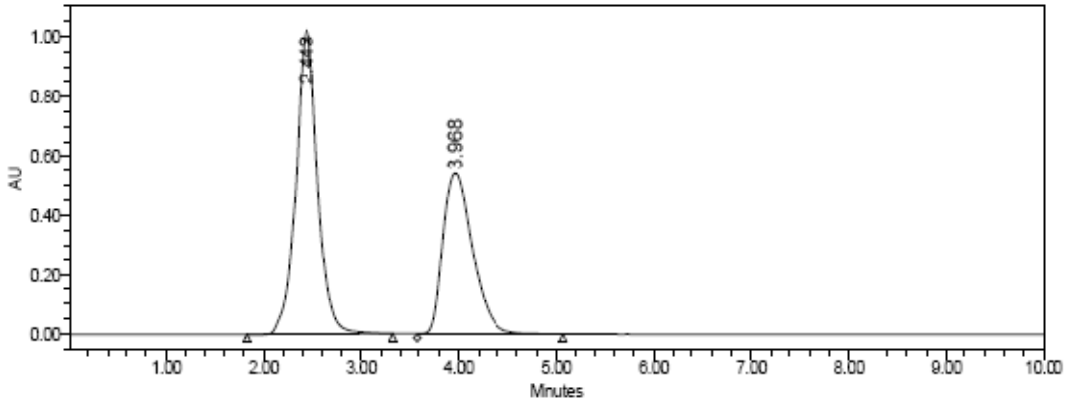


Fig No : 3.3

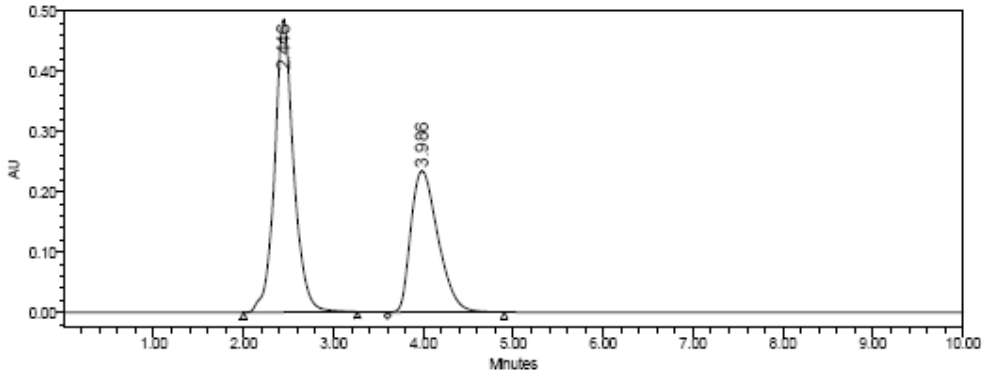


Fig No: 3.4

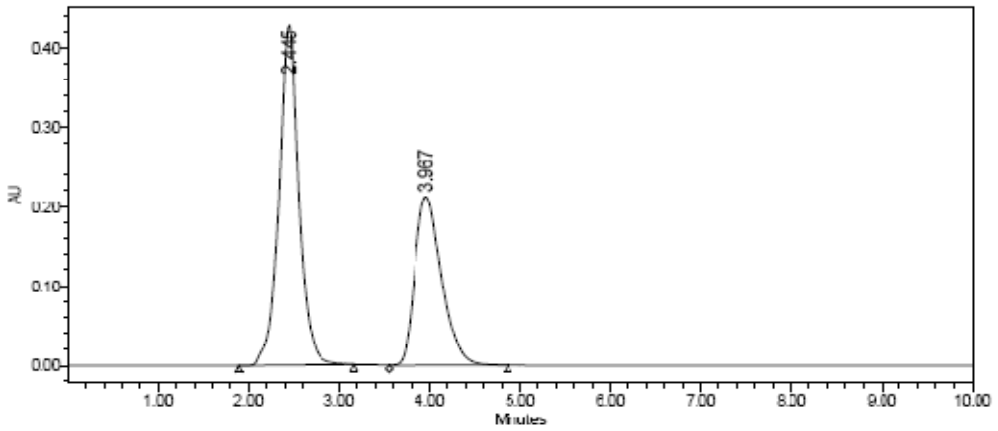


Fig No : 3.5

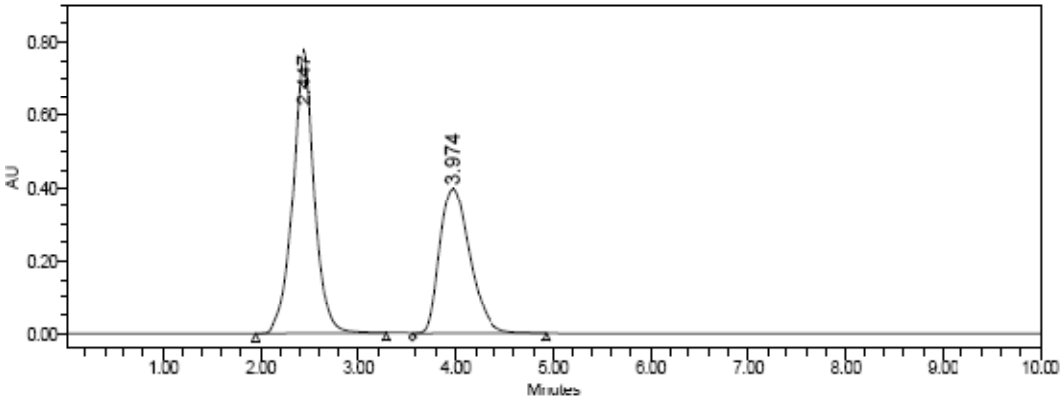


Table showing list of system suitability parameters of Lamivudine & Tenofovir.

Table No : 7

Parameters	Lamivudine	Tenofovir
Tailing factor	1.1	1.3
Retention time	2.445	3.974
Theoretical plates per unit	2766.24	2853.19

8.3 ACCURACY

The accuracy of an analytical method is the closeness of that results obtained by that method to the true value. Accuracy may often be expressed as percent recovery by the assay of known added amount of analyte.

Determination:

The accuracy of the analytical method was determined by applying the method to the analyzed samples, to which known amounts of analyte had been added. The accuracy was calculated from the test results as the percentage of analyte recovered by the assay.

Procedure:

Preparation of Standard solution (75µg/ml):

Accurately 10 mg of Lamivudine & Tenofovir were weighed and transferred into 10ml volumetric flask, about 7ml of diluent was added and sonicated for 5 minutes to dissolve it. The volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter (Stock solution).

From this 0.75ml of solution was pipetted out and transferred into 10ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Preparation of 50% sample solution:

Accurately 5mg of Lamivudine & Tenofovir were weighed and transferred into 10ml volumetric flask, about 7ml of diluent was added and sonicated for 5 minutes to dissolve it. The volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter (Stock solution).

From this 0.75ml of solution was pipetted out and transferred into 10ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Preparation of 100% solution:

Accurately 10.0mg of Lamivudine & Tenofovir were weighed and transferred 10 ml volumetric flask, about 7ml of diluent and sonicated for 5 minutes to dissolve it. The volume

was made up with mobile phase. The solution was filtered through 0.45µm membrane filter (Stock solution).

From this 0.75ml of solution was pipetted out and transferred into 10ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Preparation of 150% solution:

Accurately 15mg of Lamivudine & Tenofovir were weighed and transferred into 10ml volumetric flask, about 7ml of diluent was added and sonicated for 5 minutes to dissolve it. The volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter (Stock solution).

From this 0.75ml of solution was pipetted out and transferred into 10ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Inject 20µl of placebo and standard solutions of Accuracy -50%, Accuracy -100% and Accuracy -150% solutions into HPLC. Now calculate the amount obtained and amount added (API) for Lamivudine & Tenofovir samples.

Calculate the concentration in µg/ml in the spiked placebo in all the above cases by comparing with the standard solution. Calculate the individual recovery and mean recovery values. The chromatograms are as shown in Fig. No: 4.1, 4.2, 4.3 and the results are tabulated and shown in Table No: 8

Formula:

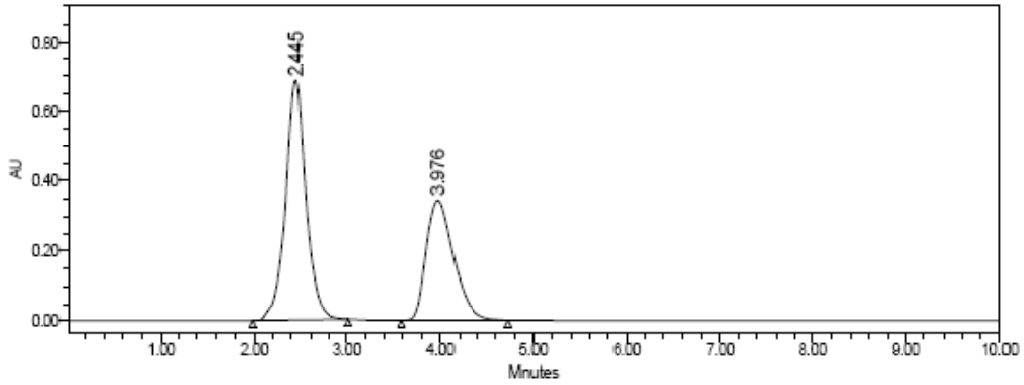
$$\%\text{recovery} = \frac{\text{(Amount recovered)}}{\text{(Actual amount added)}} \times 100$$

Acceptance criteria:

Percentage recovery in all the cases should be between 98.0 and 102.0 %.

Chromatogram showing Accuracy 50% of Lamivudine & Tenofovir.

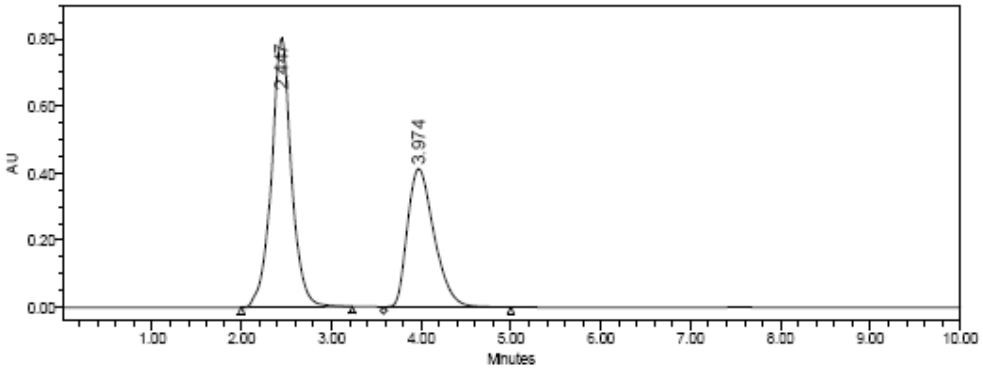
Fig No : 4.1



	Name	Retention Time (min)	Area (µV*sec)
1	Lamivudine	2.445	3340612
2	Tenofovir	3.976	2198176

Chromatogram showing Accuracy -100% of Lamivudine & Tenofovir.

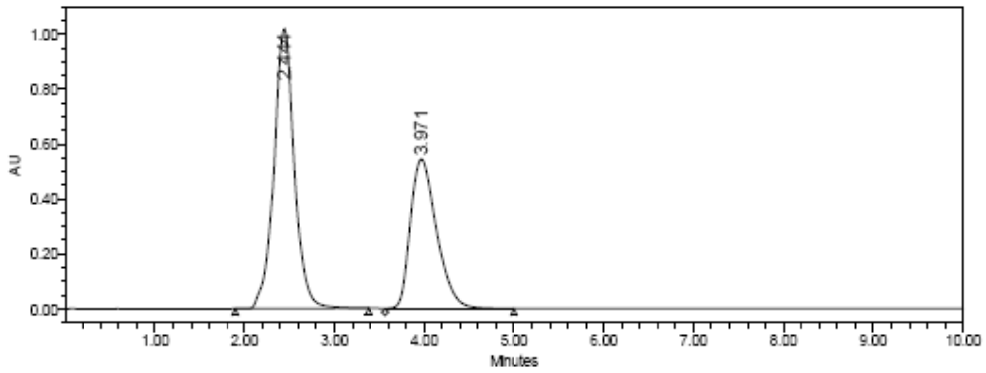
Fig No :4.2



	Name	Retention Time (min)	Area (µV*sec)
1	Lamivudine	2.447	6729401
2	Tenofovir	3.974	4602893

Chromatogram showing Accuracy – 150% of Lamivudine & Tenofovir.

Fig No : 4.3



	Name	Retention Time (min)	Area (µV*sec)
1	Lamivudine	2.444	9974837
2	Tenofovir	3.971	6895481

Table showing results of % Recovery studies for Lamivudine & Tenofovir.

Table No : 8

Inj. Sample	Spike level	Amount present	Amount recovered	% recovered	Mean recovery	Acceptance Criteria
Lamivudine	50 %	5mg	4.94mg	98.9%	98.96%	98-102%
	100 %	10mg	9.96mg	99.6%		
	150 %	15mg	14.77mg	98.4%		
Tenofovir	50 %	5mg	4.92mg	98.45%	98.84%	98-102%
	100 %	10mg	9.91mg	99.1%		
	150 %	15mg	14.87mg	98.99%		

8.4 PRECISION

Precision of an analytical method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple sampling of a homogenous sample. Precision of analytical method is usually expressed as the standard deviation and relative standard deviation.

Determination:

The precision of the analytical method was determined by assaying sufficient number of samples and relative standard deviation was calculated.

The precision of the instrument was determined by assaying the samples consecutively, number of time and relative standard deviation was calculated.

Procedure:

A) System Precision:

Preparation of Standard Solution (75 µg/ml):

Accurately 10mg of Lamivudine & Tenofovir were weighed and transferred into 10ml volumetric flask, about 7ml of diluent was added and sonicated for 5 minutes to dissolve it. The volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter (Stock solution).

From this 0.75ml of solution was pipetted out and transferred into 10ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

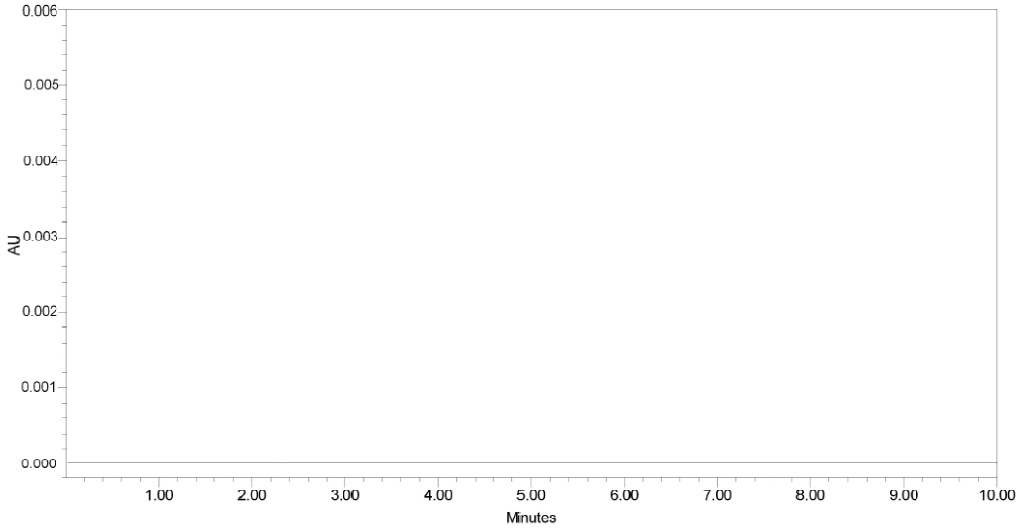
Inject 20µl of the blank solution and the standard solution of for six times and calculate the %RSD for the area of six replicate injections.The chromatograms are as shown in Fig No: 5.2-5.7 and the results are tabulated shown in Table No:9.

Blank solution: Mixture of phosphate buffer (pH3) & methanol in ratio of 40:60 was filtered and degassed.

System Precision:

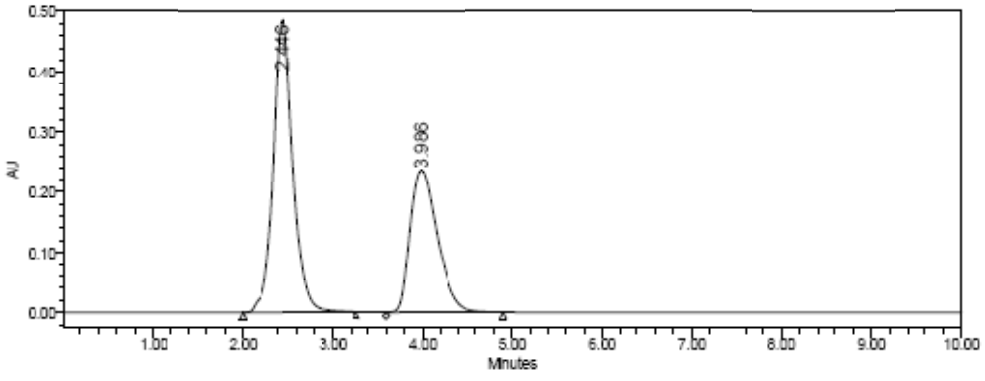
Chromatogram showing Precision-blank solution.

Fig No : 5.1



Chromatogram s+howing Precision of Test sample Preparation-1 for Lamivudine & Tenofovir

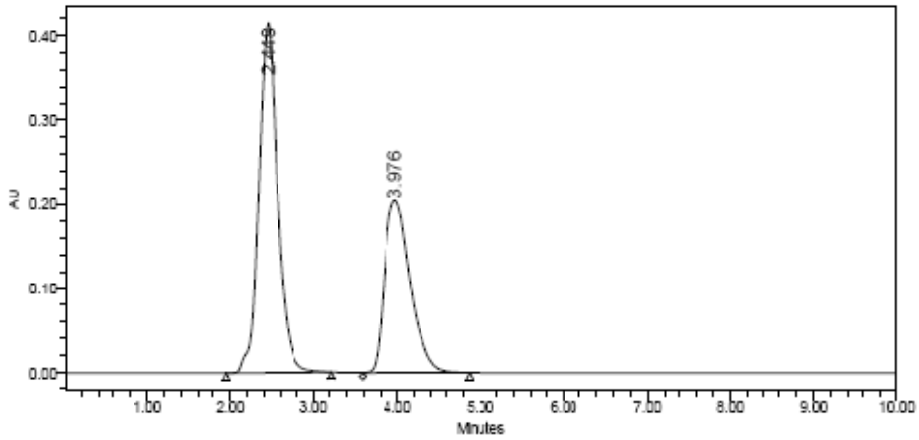
Fig No : 5.2



	Name	Retention Time (min)	Area (µV*sec)
1	Lamivudine	2.446	6852536
2	Tenofovir	3.986	4576258

Chromatogram showing Precision of Test sample Preparation-2 for Lamivudine & Tenofovir

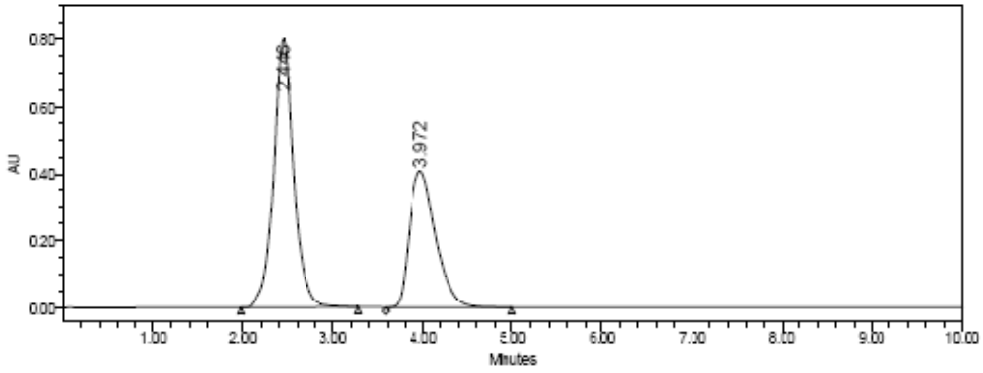
Fig No : 5.3



	Name	Retention Time (min)	Area (µV*sec)
1	Lamivudine	2.448	6926931
2	Tenofovir	3.976	4657326

Chromatogram showing Precision of Test sample Preparation-3 for Lamivudine & Tenofovir

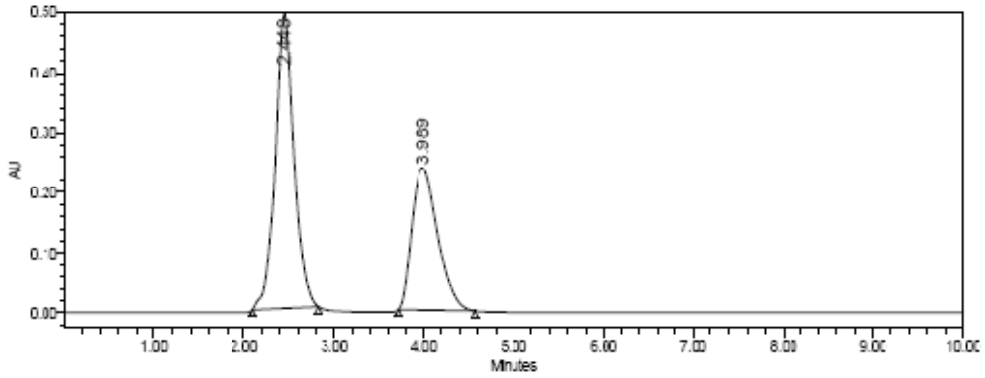
Fig No : 5.4



	Name	Retention Time (min)	Area (µV*sec)
1	Lamivudine	2.446	6795873
2	Tenofovir	3.972	4491437

Chromatogram showing Precision of Test sample Preparation-4 for Lamivudine & Tenofovir

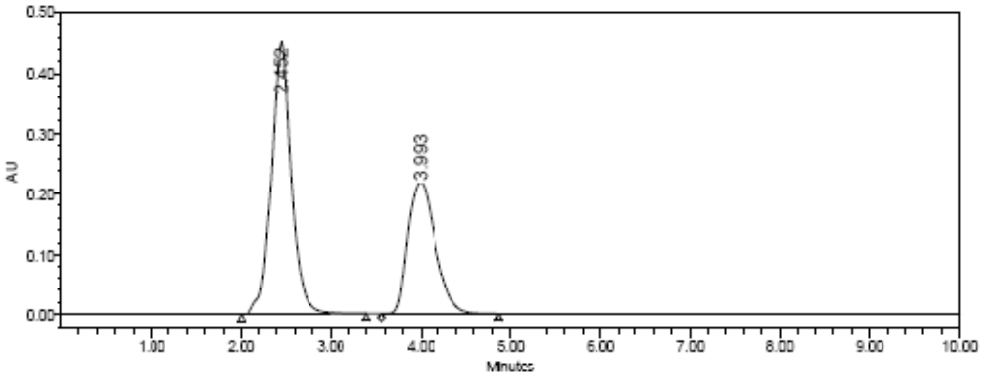
Fig No :5.5



	Name	Retention Time (min)	Area (µV*sec)
1	Lamivudine	2.448	6678317
2	Tenofovir	3.989	4713814

Chromatogram showing Precision of Test sample Preparation-5 for Lamivudine & Tenofovir

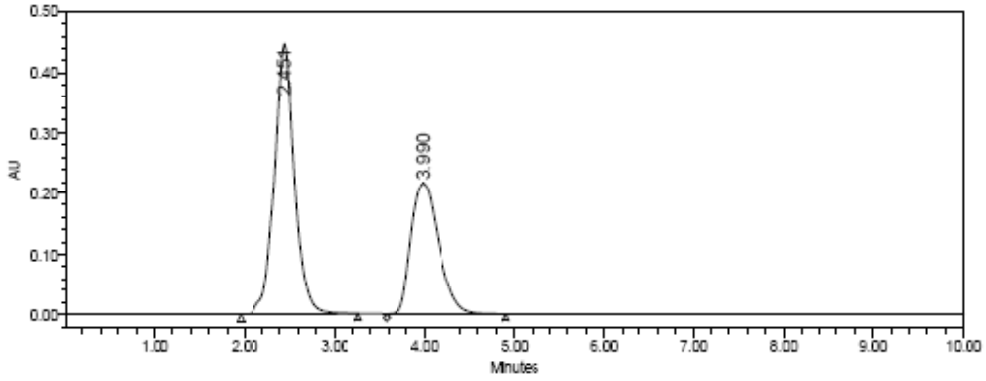
Fig No :5.6



	Name	Retention Time (min)	Area (µV*sec)
1	Lamivudine	2.452	6784739
2	Tenofovir	3.993	4713814

Chromatogram showing Precision of Test sample Preparation-6 for Lamivudine & Tenofovir

Fig No : 5.7



	Name	Retention Time (min)	Area (µV*sec)
1	Lamivudine	2.451	6807672
2	Tenofovir	3.990	4624293

B) Method Precision:

Preparation of Sample Solution:

Accurately pipette out 5ml of the sample (equivalent to 1.264gm/ml) into a 100ml volumetric flask and 70ml of diluent was added and mixed well and made up to the mark with diluent. Mix well and filter through 0.45µm filter.

Inject 50µl of the blank solution and six replicate injections of sample solution of 100 µg/ml for six times and calculate the %RSD for the area of six replicate injections.

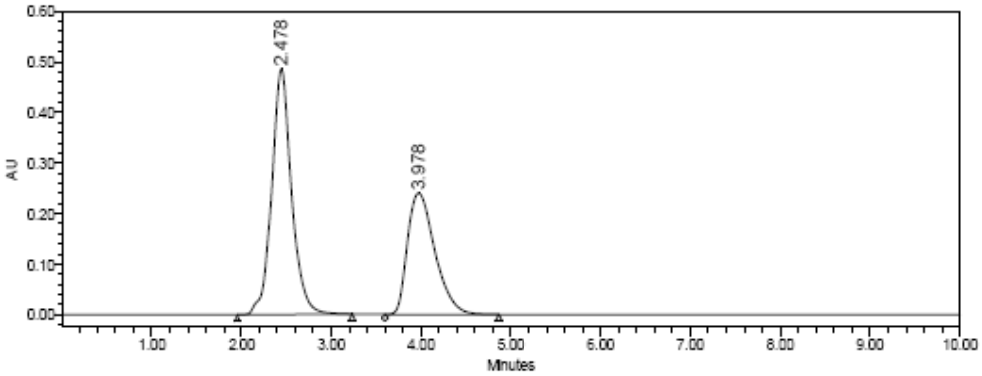
%RSD Formula: $(\sigma / \mu)*100$

Acceptance criteria: % Relative standard deviation (%RSD) for the areas of Lamivudine & Tenofovir from the standard chromatograms should not be more than 2.0

The method precision was determined by preparing the sample from the tablet formulation for five times and six successive injections of 20µl of working sample solution were injected and the chromatograms were recorded and shown in Fig No:5.8 and method precision data are shown in table. 9

Chromatogram showing Method Precision of Lamivudine & Tenofovir

Fig No : 5.8



	Name	Retention Time (min)	Area (µV*sec)
1	Lamivudine	2.478	6859856
2	Tenofovir	3.978	4396432

Table showing Results of Precision for Lamivudine & Tenofovir

Table No : 9

Parameter	**System Precision**		**Method Precision**	
Average Area	**Lamivudine**	**Tenofovir**	**Lamivudine**	**Tenofovir**
	6807678.3	4624290.8	6913809.5	4499303.3
SD	82011	80579	108767	70547
%RSD	1.20	1.74	1.57	1.56

8.5 SPECIFICITY:

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

Procedure:

Preparation of placebo :

Placebo was prepared by mixing all the excipients without active ingredients.

Accurately 28.34mg of placebo was weighed and transferred into 10ml volumetric flask, about 7ml of diluent was added and sonicated for 5 minutes to dissolve it. The volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter (Stock solution).

From this 0.75ml of solution was pipetted out and transferred into 10ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Inject 20 µl of Standard solution for six times into the HPLC system and chromatograph.

Compare the chromatograms visually and check for any interference.

Preparation of Standard solution (75µg/ml):

Accurately 10mg of Lamivudine & Tenofovir were weighed and transferred into 10ml volumetric flask, about 7ml of diluent was added and sonicated for 5 minutes to dissolve it. The volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter (Stock solution).

From this 0.75ml of solution was pipetted out and transferred into 10ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Inject 20 µl of Standard solution for six times into the HPLC system and chromatograph.

Compare the chromatograms visually and check for any interference.

Preparation of Standard + placebo:

Accurately 10mg of Lamivudine & Tenofovir were weighed and transferred into 10ml volumetric flask, about 7ml of diluents was added and sonicated for 5 minutes to dissolve it. The volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter (Stock solution).

From this 0.75ml of solution was pipette out and transferred into 10ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Inject 20 µl of Standard solution for six times into the HPLC system and chromatograph.

Compare the chromatograms visually and check for any interference. The solution was filtered through 0.45m membrane filter. The solution was injected and the reports are given in table: 10

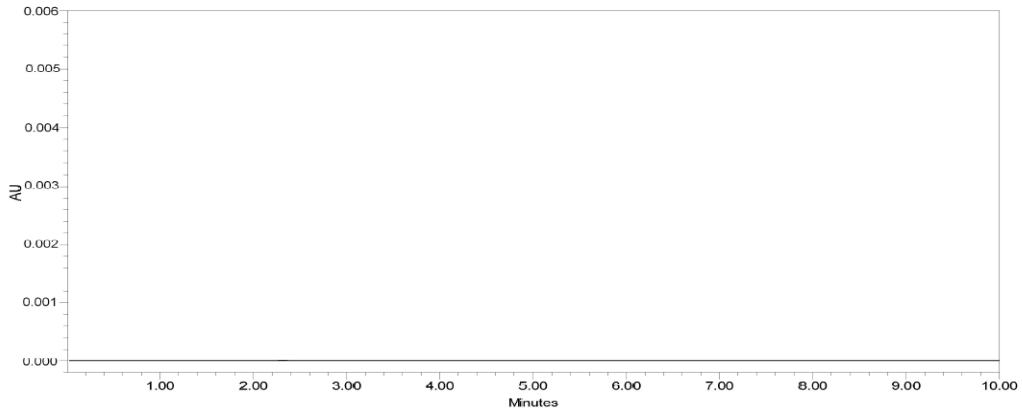
%RSD Formula: (σ /µ)*100

Acceptance criteria:

There should not be any peak in the blank and Placebo solution run at the retention time corresponding to Lamivudine & Tenofovir as in standard run.

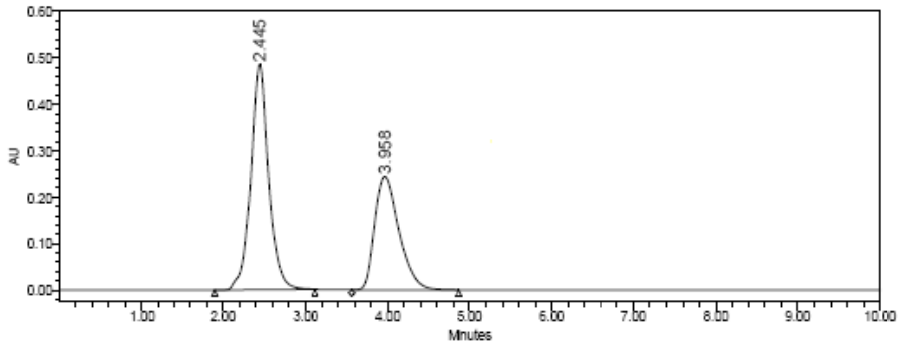
Chromatogram showing Specificity- Blank solution

Fig No : 6.1



Chromatogram showing Specificity-Standard-1 solution of Lamivudine & Tenofovir.

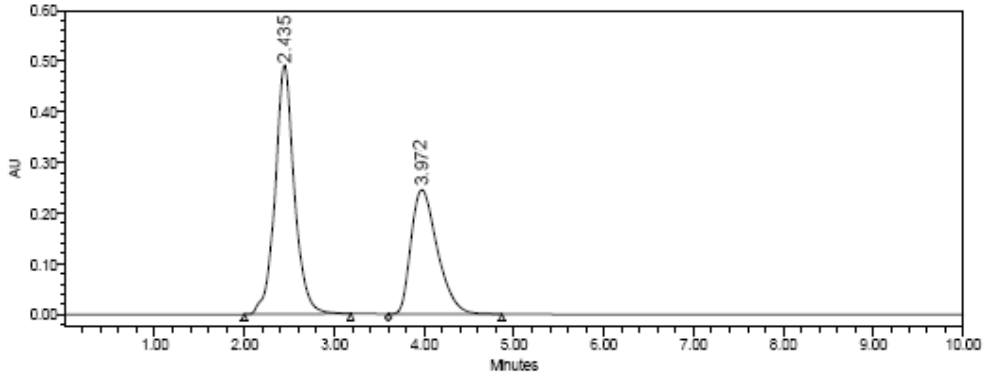
Fig No : 6.2



	Name	Retention Time (min)	Area (µV*sec)
1	Lamivudine	2.445	6673438
2	Tenofovir	3.958	6813746

Chromatogram showing Specificity- Standard-2 solution of Lamivudine & Tenofovir.

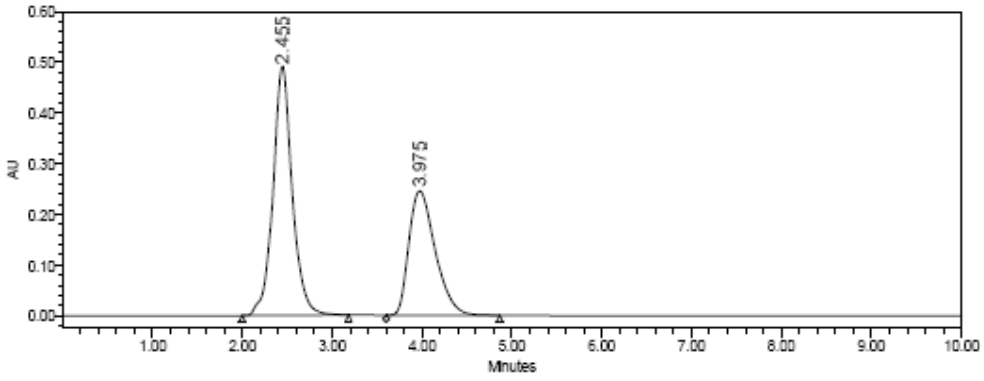
Fig No : 6.3



	Name	Retention Time (min)	Area (µV*sec)
1	Lamivudine	2.435	6813746
2	Tenofovir	3.972	4497652

Chromatogram showing Specificity- Standard-3 solution of Lamivudine & Tenofovir.

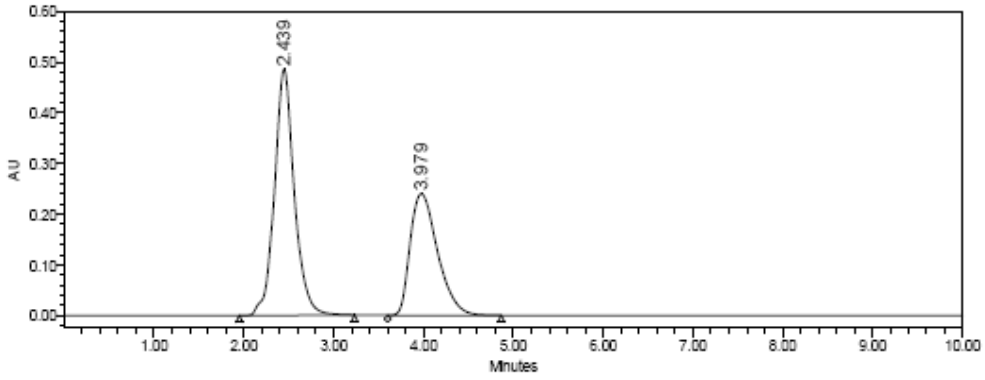
Fig No : 6.4



	Name	Retention Time (min)	Area (µV*sec)
1	Lamivudine	2.455	6793512
2	Tenofovir	3.975	4458639

Chromatogram showing Specificity- Standard-4 solution of Lamivudine & Tenofovir.

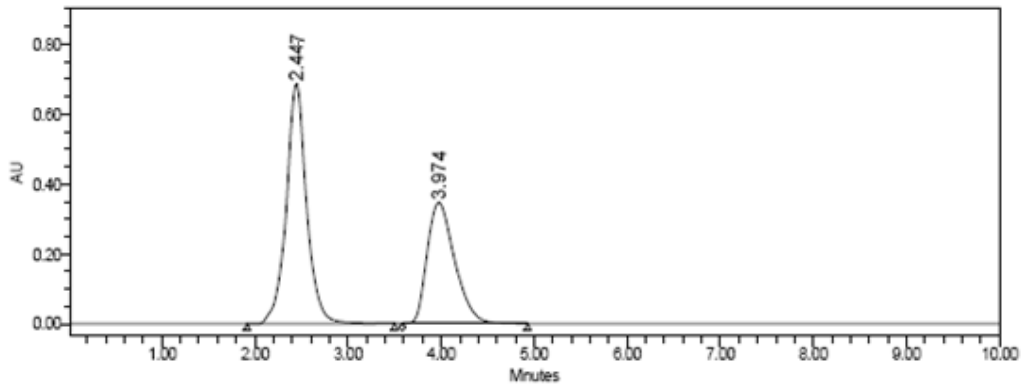
Fig No : 6.5



	Name	Retention Time (min)	Area (µV*sec)
1	Lamivudine	2.439	6634829
2	Tenofovir	3.979	4519372

Chromatogram showing Specificity- Standard-5 solution of Lamivudine & Tenofovir.

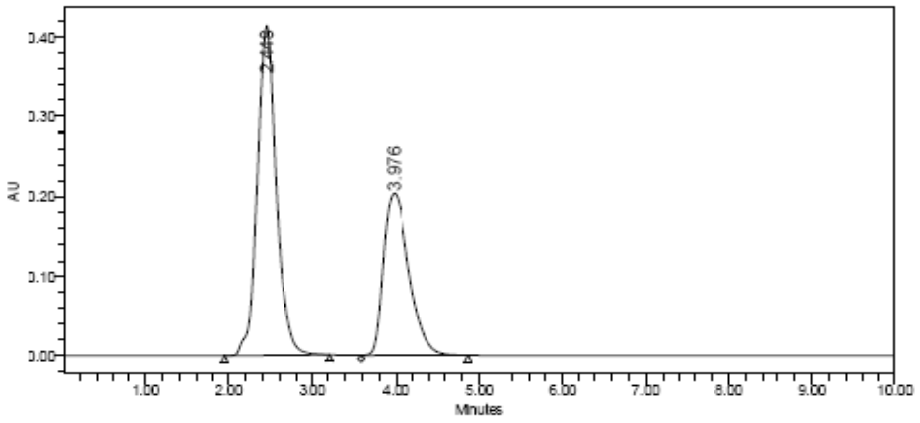
Fig No : 6.6



	Name	Retention Time (min)	Area (µV*sec)
1	Lamivudine	2.447	6769138
2	Tenofovir	3.974	4497561

Chromatogram showing Blank & Standard solution-1 overlay report of Lamivudine & Tenofovir.

Fig No : 6.7



	Name	Retention Time (min)	Area (µV*sec)
1	Lamivudine	2.448	6926931
2	Tenofovir	3.976	4657326

Table showing results of Specificity of Lamivudine & Tenofovir.

Table No : 10

Parameter	Lamivudine	Tenofovir
Average area	6736932.6	4473768.2
SD	78415	48869
%RSD	1.16	1.09

8.6 LINEARITY&RANGE

LINEARITY: Linearity is the ability of the method to obtain test results that are directly proportional to the analyte concentration within a given range.

Range: Range of analytical procedure is the interval between the upper and lower concentration of analyte in the sample (including concentrations) for which it has been demonstrated that the analytical procedure has a sutable level of precision, accuracy, and linearity.

Procedure:

Preparation of Standard Stock Solution:

Accurately 10mg of Lamivudine & Tenofovir were weighed and transferred into 10ml volumetric flask, about 7ml of diluent was added and sonicated for 5 minutes to dissolve it. The volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter (Stock solution).

Preparation of (25, 50, 75, 100 and 125 µg/ml) sample solutions:

From the above stock solution pipette out 0.25, 0.5, 0.75, 1.0 and1.25ml respectively into individual 10ml of volumetric flasks and diluted up to the mark with diluent to prepare 25,50,75,100,125,150 µg/ml of sample solutions respectively. Mix well and filter through 0.45µm filter.

Inject 20µl of blank solution and each linearity level standard solutions into the chromatographic system and measure the peak area. Plot a graph of peak area versus

concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient. The results are tabulated shown in Table No: 11

$$Correl(X,Y) = \frac{\sum (x-\bar{x})(y-\bar{y})}{\sqrt{\sum (x-\bar{x})^2 \sum (y-\bar{y})^2}}$$

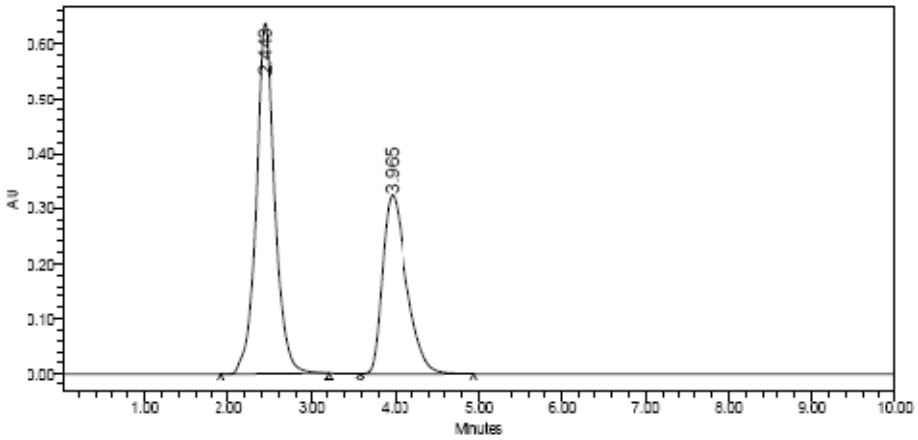
Acceptance criteria for Range:

- Correlation coefficient should not be less than 0.99%

The linearity data and analytical performance parameters of are shown in table and calibration curve of is shown Fig No: 7.6,7.7.

Chromatogram showing Linearity level-1 (25µg/ml) Lamivudine & Tenofovir.

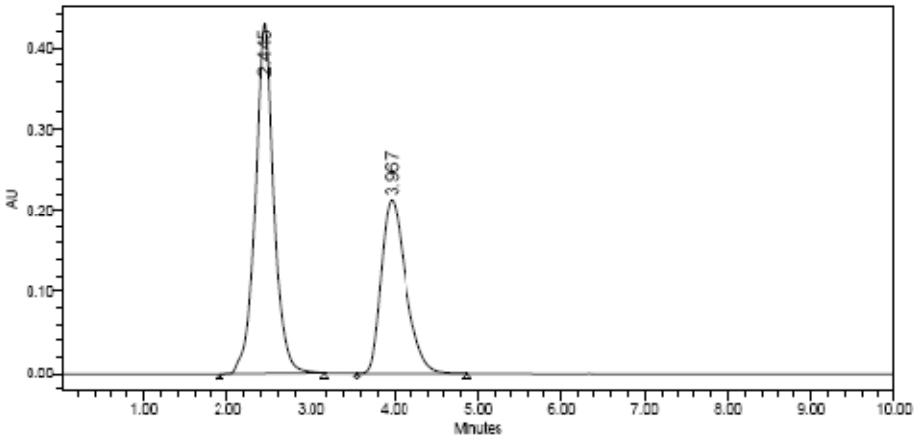
Fig No : 7.1



	Name	Retention Time (min)	Area (µV*sec)
1	Lamivudine	2.449	3266976
2	Tenofovir	3.965	2129418

Chromatogram showing Linearity level-2 (50µg/ml) of Lamivudine & Tenofovir.

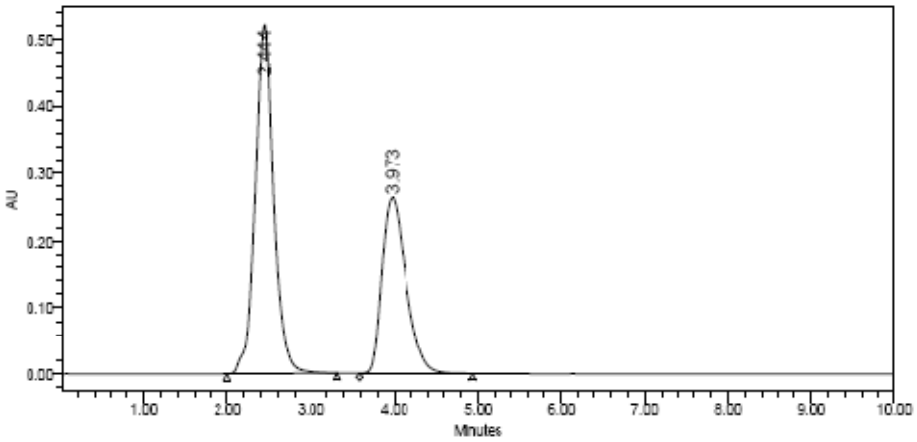
Fig No: 7.2



	Name	Retention Time (min)	Area (µV*sec)
1	Lamivudine	2.445	4954694
2	Tenofovir	3.967	3294157

Chromatogram showing Linearity level-3 (75µg/ml) of Lamivudine & Tenofovir.

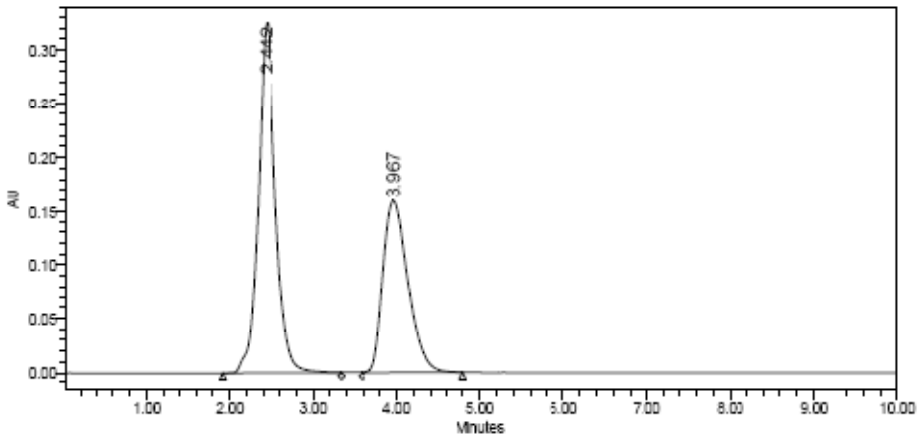
Fig No : 7.3



	Name	Retention Time (min)	Area (µV*sec)
1	Lamivudine	2.444	6739592
2	Tenofovir	3.973	4458836

Chromatogram showing Linearity level-4 (100µg/ml) of Lamivudine & Tenofovir.

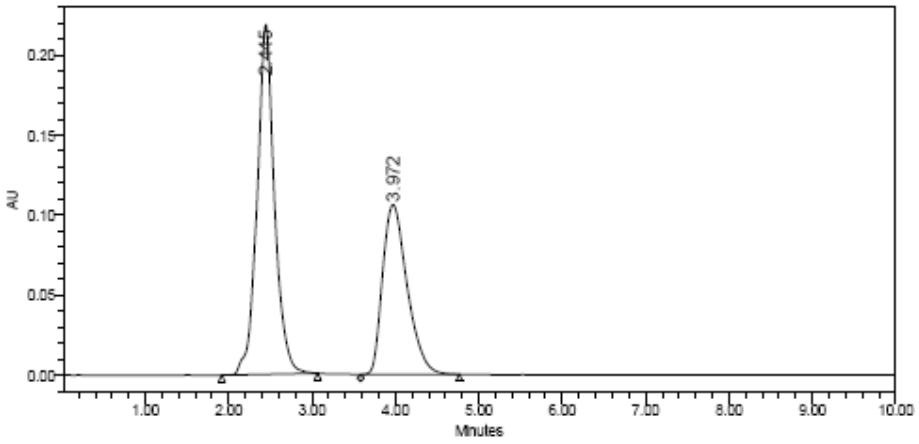
Fig No : 7.4



	Name	Retention Time (min)	Area (µV*sec)
1	Lamivudine	2.442	8524491
2	Tenofovir	3.967	5673545

Chromatogram showing Linearity level-5 (125g/mlµ) of Lamivudine & Tenofovir.

Fig No : 7.5



	Name	Retention Time (min)	Area (µV*sec)
1	Lamivudine	2.445	10209389
2	Tenofovir	3.972	6838247

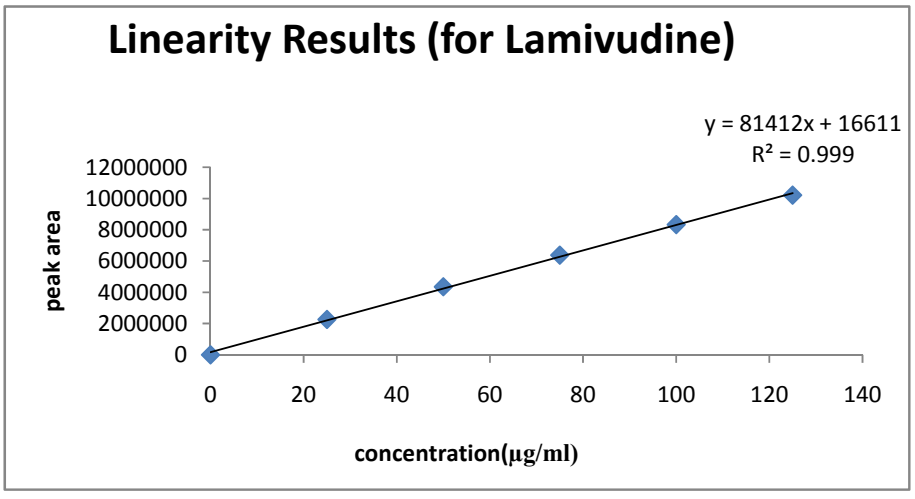
Linearity Results (for Lamivudine):

Table No : 11

S. No	Linearity Level	Concentration	Area
1	I	25µg/ml	2266976
2	II	50µg/ml	4954694
3	III	75µg/ml	6730592
4	IV	100µg/ml	8384491
5	V	125µg/ml	10209389
Correlation Coefficient			0.999

Figure showing Linearity graph of Lamivudine.

Fig No : 7.6



The correlation coefficient value was found to be 0.999

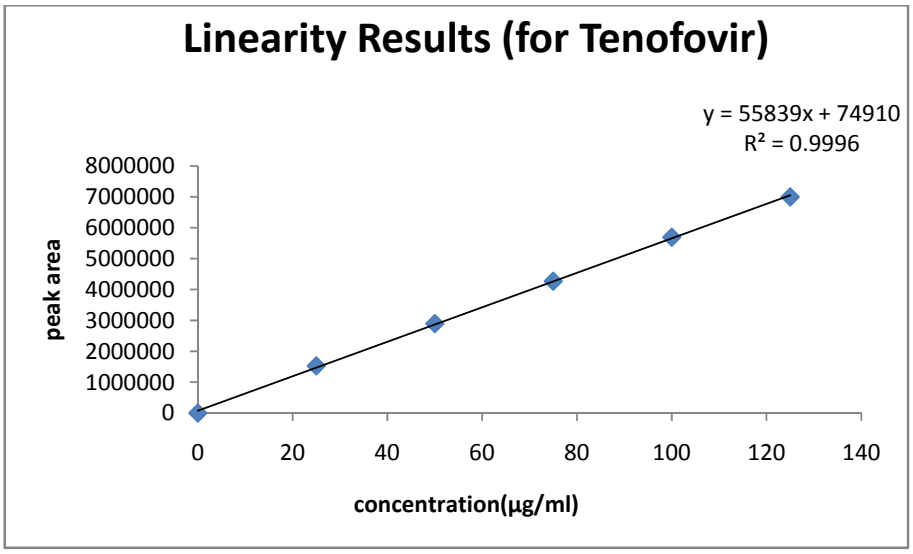
Linearity Results for Tenofovir:

Table No : 12

S. No	Linearity Level	Concentration	Area
1	I	25µg/ml	1529418
2	II	50µg/ml	2898127
3	III	75µg/ml	4568836
4	IV	100µg/ml	5693545
5	V	125µg/ml	6999247
Correlation Coefficient			0.9996

Figure showing linearity graph of Tenofovir.

Fig No : 7.7



The correlation coefficient value was found to be 0.9996

8.7 ROBUSTNESS

It is a measure of ability to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

The robustness of an analytical method was determined by analysis of aliquots from homogenous lots by differing physical parameters that may differ but were still within the specified parameters of the assay for example change in physical parameters like flow rate, column temperature and mobile phase ratio.

A) the flow rate was varied at 0.7 to 0.9 ml/min.

Preparation of Standard Solution (75µg/ml):

Accurately 10mg of Lamivudine & Tenofovir were weighed and transferred into 10ml volumetric flask, about 7ml of diluent was added and sonicated for 5 minutes to dissolve it. The volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter (Stock solution).

From this 0.75ml of solution was pipetted out and transferred into 10ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Inject 20µl of the blank solution and the standard solution of 75 µg/ml for five times and analysed using varied flow rates (0.7ml, 0.9 ml) along with method flow rate and calculate the %RSD for the area of five replicate injections.

a) The flow rate was varied at ±0.1 ml/min.

%RSD of Lamivudine & Tenofovir assay under these conditions is calculated and the results are shown in below.

PROCEDURE

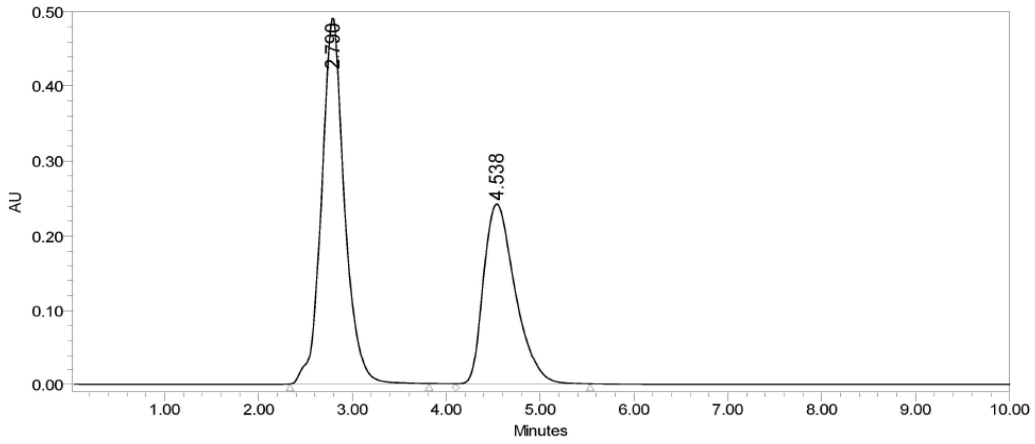
a) The flow rate was varied at ±0.1 ml/min.

Standard solution 70 µg/ml was prepared and analysed using the varied flow rates along with method flow rate and the chromatograms were recorded. The results are shown in table 11.

Flow variation-1 (0.7mL/min):

Chromatogram showing Robustness-Flow variation (0.7ml/min) of Lamivudine & Tenofovir.

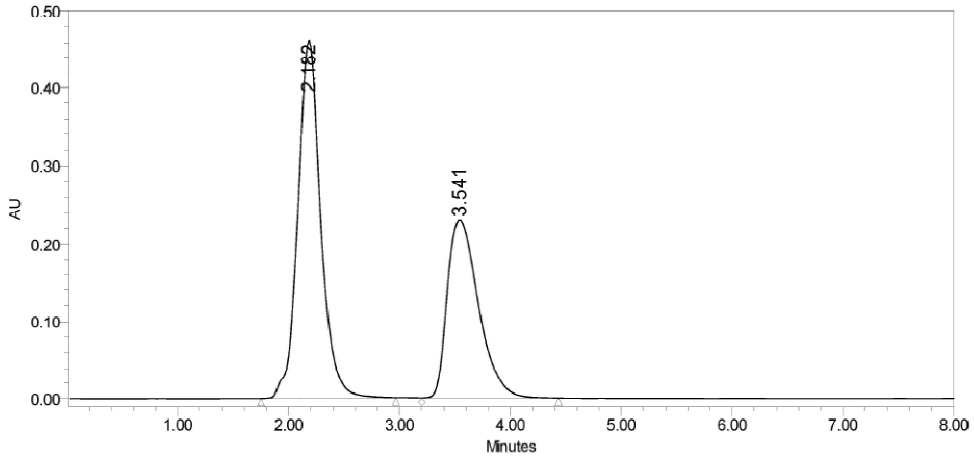
Fig No : 8.1



	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing	USP Resolution
1	Lamivudine	2.79	8344338	491790	2671.86	1.1	
2	Tenofovir	4.54	5675524	242492	2876.79	1.4	3.20

Robustness flow rate (0.9ml):

Fig No :8.2



	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing	USP Resolution
1	Lamivudine	2.18	6590717	462877	2575.82	1.1	
2	Tenofovir	3.54	4500085	230878	2800.83	1.5	3.05

B) Mobile phase

Preparation of Standard Solution (75µg/ml):

Accurately 10mg of Lamivudine & Tenofovir were weighed and transferred into 10ml volumetric flask, about 7ml of diluent was added and sonicated for 5 minutes to dissolve it. The volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter (Stock solution).From this 0.75ml of solution was pipetted out and transferred into 10ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Inject 20µl of the blank solution and the standard solution of 75 µg/ml for five times and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method and calculate the %RSD for the area of five replicate injections. The chromatograms are as shown in Fig No: 40 and the results are tabulated shown in Table No: 16.

%RSD Formula: (σ /µ)*100

Acceptance criteria:

Relative standard deviation (RSD) of areas of Lamivudine & Tenofovir from five standard chromatograms in all the flow rate variation and mobile phase composition should not be more than 2.0 %.

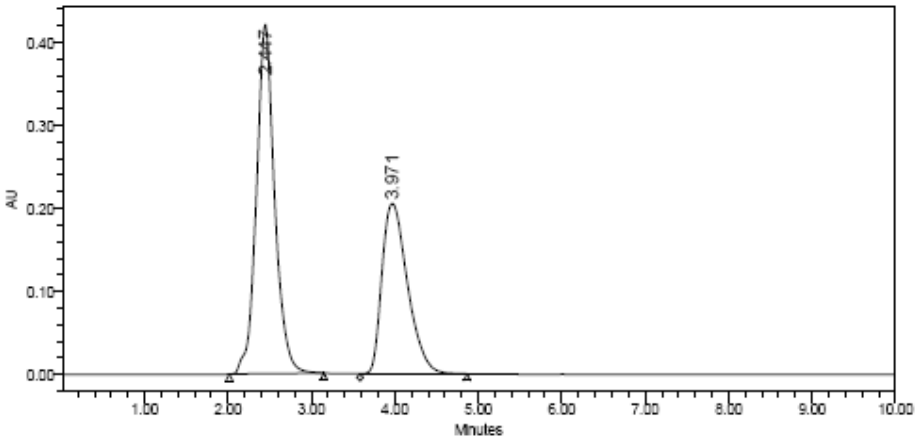
The Organic composition in the Mobile phase was varied from ±5%:

Standard solution 70 µg/ml was prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method and the chromatograms were recorded.

The graphs are shown in fig 8.3and 8.4 and the results are shown in table no : 13 .

ROBUSTNESS MOBILE PHASE (35:65)

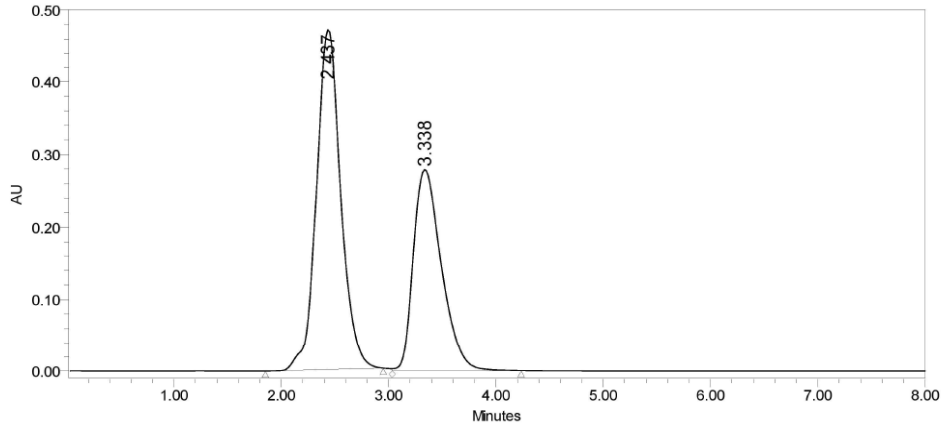
Fig No :8.3



	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing	USP Resolution
1	Lamivudine	2.44	6329717	459765	2652.12	1.2	
2	Tenofovir	3.97	3990067	229765	2795.62	1.4	3.15

ROBUSTNESS MOBILE PHASE (45:55)

Fig No: 8.4



	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing	USP Resolution
1	Lamivudine	2.44	7408362	471253	2580.24	1.0	
2	Tenofovir	3.34	5077859	279210	2785.37	1.4	1.96

Table showing Robustness results for change in flow rate and mobile phase of Lamivudine & Tenofovir.

Table No : 13

Flow rate	Inj. Sample	Area	Plate count	Tailing	RT
0.7ml/min	**Lamivudine**	8344338	2671.86	1.1	2.79
	Tenofovir	5675524	2876.79	1.4	4.54
0.9ml/min	**Lamivudine**	6590717	2575.82	1.1	2.58
	Tenofovir	4500085	2800.83	1.5	3.54
Mobile phase variation					
35:65	**Lamivudine**	6329717	2652.12	1.2	2.44
	Tenofovir	3990067	2795.62	1.4	3.97
45:55	**Lamivudine**	7408362	2580.74	1.0	2.44
	Tenofovir	5077859	2785.37	1.4	3.34

8.8 RUGGEDNESS

Ruggedness as the degree of reproducibility of test result obtained by the analysis of the same of the samples under verity of normal test conditions, such as different labs, different analyst, and different lots of reagents, different elapsed assay times, different assay temperature, different days etc.

Ruggedness is normally expressed as the of influence on test results of operational and environmental variables of the analytical method.

Procedure:

Preparation of Standard Solution (75µg/ml):

Accurately 10mg of Lamivudine & Tenofovir were weighed and transferred into 10ml volumetric flask, about 7ml of diluent was added and sonicated for 5 minutes to dissolve it. The volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter (Stock solution).

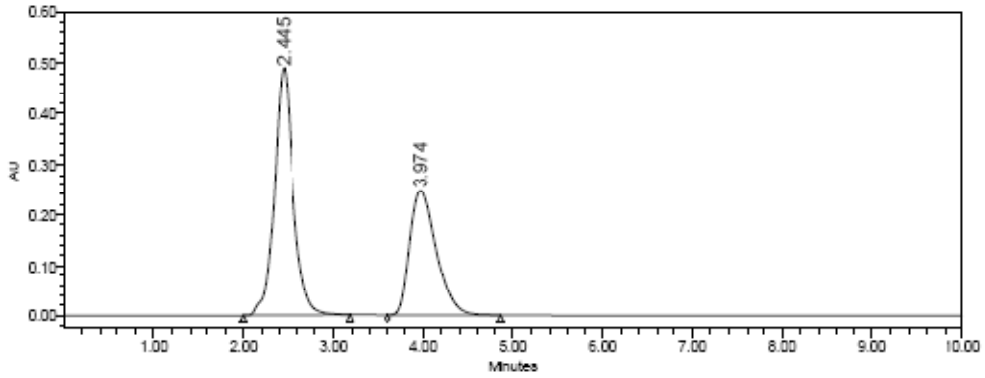
From this 0.75ml of solution was pipetted out and transferred into 10ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Inject the blank solution and standard solution of 75µg/ml for six times and analysed by two analysts and calculate the %RSD for the area of injections.

Ruggedness of the current method was demonstrated by analyzing two samples (assay) of tablet formulation by two analysts in the same laboratory on two different days. The chromatograms are as shown in Fig.No:9.1-9.4 and the results are as shown in the Table No:14 and15 indicating the ruggedness of the method.

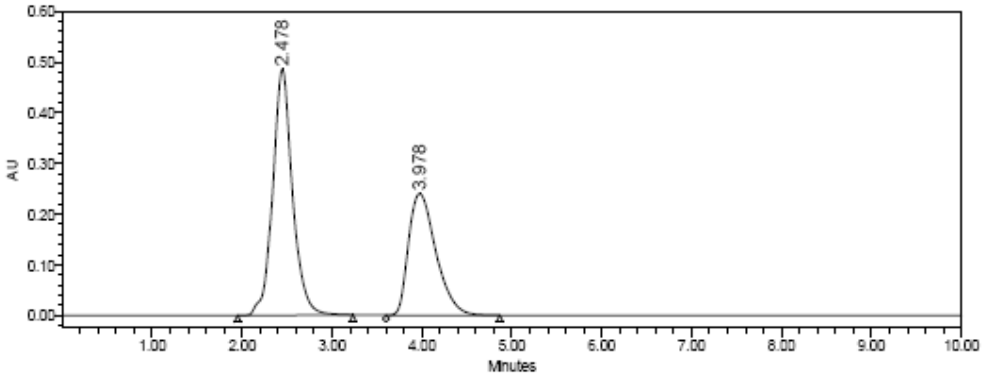
Chromatogram showing Ruggedness: Day-1, Analyst-1 of Lamivudine & Tenofovir

Fig No : 9.1



	Name	Retention Time (min)	Area (µV*sec)
1	Lamivudine	2.445	6698915
2	Tenofovir	3.974	4483569

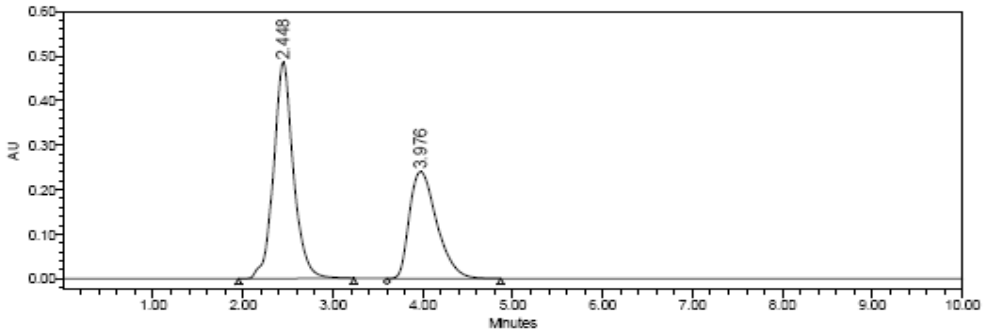
Fig No : 9.2



	Name	Retention Time (min)	Area (µV*sec)
1	Lamivudine	2.478	6713468
2	Tenofovir	3.978	4439786

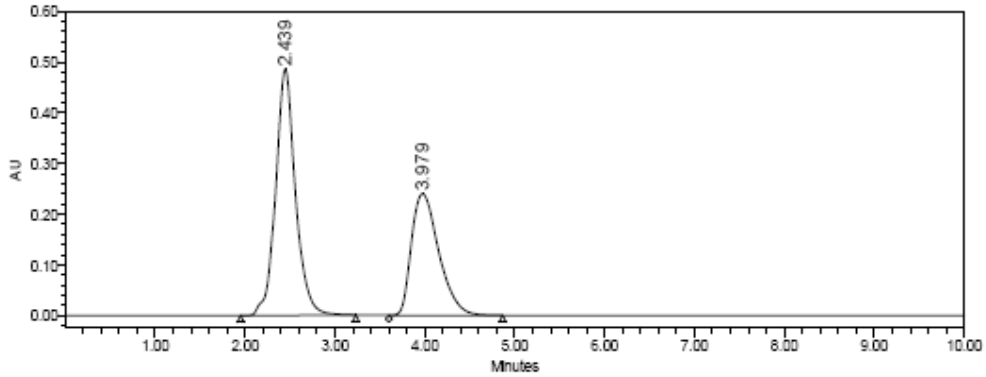
Chromatogram showing Ruggedness: Day-2, Analyst-2 of Lamivudine & Tenofovir.

Fig No : 9.3



	Name	Retention Time (min)	Area (µV*sec)
1	Lamivudine	2.448	6729853
2	Tenofovir	3.976	4487653

Fig No :9.4



	Name	Retention Time (min)	Area (µV*sec)
1	Lamivudine	2.439	6734821
2	Tenofovir	3.979	4396175

Chromatogram showing Ruggedness: different columns, instruments, analyst of Lamivudine & Tenofovir

Table No : 14

S.No	Column Code	Instrument Code	Analyst	Result Obtained (%)	
				Lamivudine	Tenofovir
1.	C-01	W-29	I	99.5%	99.25
2.	C-02	W-30	II	99.85%	99

Table No : 15

Parameter	Result observed		Acceptance Criteria
	Lamivudine	Tenofovir	
Percentage Content	99.67	99.12	98 – 102%

9.1 SUMMARY

Method development:

The developed method has an advantage of Lamivudine & Tenofovir in RP-HPLC. The following tables give the results of the method development quantitation and validation parameters.

FIXED CHROMATOGRAPHIC CONDITION

Table No: 16

OPTIMIZED CHROMATOGRAPHIC CONDITIONS	
Mode of separation	isocratic elution
Mobile phase	Solvent-A: Phosphate buffer pH-3 Solvent-B: methanol
Column	Symmetry C8 (4.6 x 150mm, 5 µm, Make: ACE)
Flow rate	0.8 mL/ min
Detection Wavelength	260 nm
Injection volume	20 µl
Column oven temperature	Ambient(30°C)
Run time	10 min

Quantitative Estimation

Table No: 17

S. No	Compound name	Assay value
1.	Lamivudine	99.56%
2.	Tenofovir	99.1%

Acceptance criteria: 98-102%

Table showing Summary of results of method validation for Lamivudine & Tenofovir

Table no: 18

S. No	Parameter	Requirement	results		Acceptance criteria
			Lamivudine	**Tenofovir**	
1.	System suitability	RT	2.445	3.99	
2.		Tailing factor	1.1	1.3	NMT 2
3.					
4.		Plate count	2766.24	2853.19	NLT 2000
5.		Assay value	99.56%	99.1	98-102%
6.	Accuracy	% Recovery	98.965	98.84%	98-102%
7.	Precision	%RSD	1.20	1.74	NMT 2%
8.	Specificity	No interference	Pass	pass	No interference
8.	Linearity	Correlation coefficient	0.999	0.999	NLT 0.999
9.	Range	Concentration	25-125µg/ml	25-125µg/ml	Nil
10.	Robustness	%RSD	1.37	1.56	NMT 2%
11.	Ruggedness	%RSD	99.67	99.12	98-102%

9.2 CONCLUSION

- A new method is developed for Simultaneous Estimation of Lamivudine and Tenofovir by RP-HPLC method. The sample preparation is simple and the analysis time is short. The analytical procedure is validated as per ICH Q2B guidelines and shown to be accurate, precise and specific.

- This method represents a fast analytical procedure for the simultaneous quantization of Lamivudine and Tenofovir. The method is amenable to the routine analysis of large numbers of samples with good precision and accuracy.

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